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CEREAL CHEMISTRY

VOL. XXIV JANUARY, 1947 No. 1

COMMERCIAL PRODUCTION AND USE OF MOLD BRAN

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(Presented at the Annual Meeting, May 1946; received for publication July 29, 1946)

The purpose of this paper is to present some of the laboratory, pilot plant, and semicommercial investigations which have led to the successful commercial production of mold bran, a primary fungal amylase preparation. The pioneering research was conducted by the authors and their collaborators in four laboratories, Iowa State College, University of Idaho, University of Nebraska, and Farm Crops Processing Corporation, over a period of nearly 10 years. Space permits presentation here of only the most salient points.

The use of amylases in industrial processes has long been of interest to cereal chemists. Although barley malt amylases have been most generally used in industry, amylase concentrates prepared from fungal or bacterial sources have also found limited use in a number of industrial processes. The amylases from various sources differ in properties, such as extent of liquefaction, dextrinization and saccharification of starch, temperature for optimum activity, thermal stability, and optimum pH range. These differences in properties have occasioned much interest in recent years and investigations have been made to determine more accurately the special properties of cereal, bacterial, and fungal amylases in order to be able to select or adapt the most favorable for specific industrial uses. Research has also been active in an effort to develop more efficient and cheaper methods for the production of bacterial and fungal amylase preparations of high activity.

The largest industrial use of amylases is for the saccharification of starch for the production of alcohol by fermentation of grains and potatoes. For alcoholic fermentation of starchy substrates in occidental countries, barley malt has most generally been used as the sac-

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charifying agent, whereas in the Orient fungal amylases have been most extensively employed. Suggestions have frequently been made that fungal amylases be substituted for malt in the alcoholic fermentation of grains in America and in Europe, but developments along this line have not been significant, particularly in America. In Europe the *amylo* process, which uses fungal amylases produced by growing selected molds in the grain mash prior to yeast fermentation, has been employed to a limited extent. Grove (1914) and Owen (1933) have described this process in some detail.

Takamine (1914) first suggested the use of a product prepared by growing the mold *Aspergillus oryzae* on wheat bran to replace malt in alcoholic fermentation of grains, but without significant industrial developments. This idea was recently revived by Underkofler, Fulmer, and Schoene (1939), and favorable results in the use of such fungal preparations, designated as mold bran, have been reported by these authors, by Hao, Fulmer, and Underkofler (1943), by Roberts, Laufer, Stewart, and Saletan (1944), and by Hao and Jump (1945) on the laboratory scale. Beresford and Christensen (1941) reported the successful use of mold bran in the experimental alcohol plant of the University of Idaho, yields of alcohol from potatoes being somewhat better with mold bran than with malt. Recently Underkofler, Severson, and Goering (1946) reported extensive commercial tests in which yields of alcohol were at least as good or somewhat better with mold bran as with malt.

In order for mold bran to be accepted in the alcohol fermentation industry, or in other industries employing amylases, it must possess high amylolytic activity and be readily available at lower relative cost than barley malt. That is, a cheap and efficient method of commercial production is required. The research reported in this paper has resulted in such successful commercial application.

Laboratory Investigations

Takamine (1913, 1914, 1918) recommended the use of a rotating drum for the production of mold bran on an industrial scale. In his process the bran was steamed, inoculated with spores of the mold *Aspergillus oryzae*, and incubated in a slowly rotating drum through which humidified air was passed. Takamine experienced considerable difficulty with contamination by undesirable microorganisms, especially bacteria. In an effort to minimize such contamination he (1913a, 1914, 1915) attempted to acclimatize the mold culture to concentrations of formaldehyde sufficient to prevent growth of the undesirable contaminants.

During the early laboratory work of the authors and their co-

workers, the drum method of Takamine was employed, and has been described in detail by Underkofler, Fulmer, and Schoene (1939). It was found during the course of this early work that use of dilute hydrochloric acid, rather than water, to moisten the bran gave better results. By acidifying the bran with 0.1 to 0.3 *N* hydrochloric acid to a pH of 3.5 to 4.5, sterilization was improved, and the presence of the acid inhibited the growth of undesirable microorganisms during the growth period of the mold. Underkofler, Fulmer, and Schoene (1939) described this development, and the use of acid for this purpose was patented by Underkofler (1942) and by Christensen (1944).

In going from the 5-gallon laboratory drums to larger drums, difficulty was experienced in securing good growth of the mold, owing to destruction of the delicate mold mycelium in the early growth stages by the tumbling of the bran particles. The larger the drum, the greater was the difficulty experienced. Hence, one of the authors, at the University of Idaho, developed a laboratory method involving the incubation of the mold in a small covered pan having a perforated bottom permitting air to be forced through the bran mass. A modification of this procedure was described in detail by Hao, Fulmer, and Underkofler (1943), and this method has been employed in most of the laboratory work. In this procedure the bran is moistened, sterilized, inoculated with mold spores, and held in the covered pan. Air is passed through the bran in the pan at such rate that the temperature is kept at 35° to 40°C, the direction of aeration being reversed at hourly intervals by alternately applying pressure and suction. After 24 hours the contents are removed from the pan, broken into pieces 1 inch in diameter or less, placed on a table in a pile about 2 inches deep, and covered with a damp cloth. It is normally held for another 24 hours, and is then dried at room temperature.

During the course of the work at the University of Idaho it was found desirable to add to the bran traces of salts of iron, zinc, and copper and of phosphates. At Iowa State College it was found that with some wheat brans the addition of these materials had little effect on the rate or extent of the growth of *A. oryzae* or on the amylolytic activity of the resulting mold bran. On the other hand, with certain brans addition of these materials resulted in considerably better mold growth and higher amylolytic activity. The use of such mineral salts and phosphates was patented by Christensen (1944).

Hao, Fulmer, and Underkofler (1943) investigated the saccharifying ability, for alcoholic fermentation mashes, of mold bran preparations produced by 27 different strains of molds. Several strains of the species *A. oryzae*, *Rhizopus delemar*, and *R. oryzae* gave products which were almost equal in saccharifying ability as measured by fer-

mentation tests on corn mashes. It was concluded, however, that strains of *A. oryzae* were most satisfactory because of superior cultural characteristics, including more abundant sporulation and denser mycelial growth.

Another method which was tested on the laboratory scale for the preparation of mold bran was incubation on trays. Excellent growth of the mold resulted in such tests conducted at the University of Idaho and the University of Nebraska. The bran was simply mixed with dilute acid, sterilized, cooled at about 35°C, inoculated with spores of *A. oryzae*, placed on the trays in thin layers, and incubated at 30° to 35°C in cabinets in which humidified air was circulated. The tray method was employed on a pilot plant scale at the experimental alcohol plant of the University of Idaho for the production of mold bran in 100-pound lots.

The mold bran produced by any of the laboratory procedures was dried by spreading it out on the table top and allowing it to air-dry at room temperature. If piled in thick layers, or if allowed to remain in chunks larger than about one inch in diameter, the moist material heats rapidly, resulting in autolysis of the mold and destruction of the enzymes present. It has been found that the amylase potency for saccharifying fermentation mashes does not decrease to a measurable extent even after storage of the dry mold bran for periods up to 24 months. The critical moisture level for storage without deterioration is about 15%. It was found in the laboratory that the mold bran can be used equally well for saccharifying grain mashes either in the moist condition directly after production or after drying the material. In either case it is advisable to prepare a slurry of the mold bran in water before use. There is no advantage in grinding the mold bran to reduce the particle size.

Throughout the research there has been a continued effort to find a rapid laboratory test that would serve to evaluate the mold bran as well as other saccharifying agents, that is, a method to indicate the amount of saccharifying agent required and the alcohol yield that could be expected. However, no method except actual fermentation tests on a series of mashes with several levels of the saccharification agent has given satisfactory results. Thorne, Emerson, Olson, and Peterson (1945) came to a similar conclusion with respect to the evaluation of malts for alcohol production, and Kneen (1945) has emphasized the deficiency of customary methods for evaluating starch-degrading properties for fermentation purposes. Hence, a fermentation procedure was used for the final evaluation of all the mold bran preparations. Standardized conditions were used, and the results were controlled by comparison with fermentations of mashes saccharified with malt.

A great deal of research was required to develop a satisfactory method for growing spore cultures on bran to serve as inoculum. It was found that if, during the incubation period, the bran dries too rapidly, poor spore production is obtained; if the bran does not dry rapidly enough after sporulation is complete, autolysis occurs, with a rapid loss in spore viability. A good dry spore culture of *A. oryzae* is a greenish color, not brown or black, and a dense cloud of spores arises when the flask containing it is shaken. Spore cultures are best prepared by mixing materials in the proportions of 10 g ground corn, 100 g wheat bran, and 60 ml 0.2 *N* hydrochloric acid containing 0.62 ppm $ZnSO_4 \cdot 7H_2O$, 0.63 ppm $FeSO_4 \cdot 7H_2O$, and 0.08 ppm $CuSO_4 \cdot 5H_2O$, distributing the moist material in 10-g quantities in 250-ml flasks and sterilizing in the autoclave. The cooled bran is then inoculated with the mold spores, the bran distributed on one side of the flask by gentle tapping, and the flask incubated at 30°C while lying on the side. The presence of corn meal and salts results in more rapid growth and sporulation of the mold and tends to control the rate of drying so that well-sporulated cultures uniformly result. In the laboratory, about 1% of dry spore culture was used in inoculating the moist, sterile bran for producing mold bran.

Pilot Plant Investigations

During the early war period when the shortage of malt began to appear, a pilot plant research project was established by the Office for Production Research and Development of the War Production Board for the purpose of developing feasible methods for the commercial production of mold bran. In the laboratory investigations a number of problems had been encountered, the solution of which was imperative in order to translate the laboratory methods to the plant scale. The principal problems were the following: (1) methods for handling the bulky bran, for mixing it with dilute acid, and for cooking and sterilizing it; (2) methods for growing spore cultures for inoculum in large amounts; (3) methods for inoculation and the proportion of inoculum to be employed; (4) methods for incubation during the growing period; (5) methods for usage and storage of the finished mold bran. The pilot plant research is discussed below under these headings.

1. Mixing, Cooking, and Sterilizing. For large-scale production of mold bran an efficient system for mixing the bran with the dilute acid and for cooking the bran was needed. Pressure sterilization of large masses of wet bran in bulk presents serious problems. It was believed that the most feasible method for cooking to insure thorough sterilization of the bran was by direct steam injection in a unit which provided

for continuous agitation so that the moist bran particles would be in constant direct contact with the steam. Experience during the pilot plant operations in the experimental alcohol plant of the University of Idaho had shown that when acid was employed for moistening the bran it was only necessary to hold the bran at 93° to 99°C for 15 to 30 minutes to obtain practical sterility. A few mold spores survived such treatment but caused no serious trouble. Butyric acid bacteria,

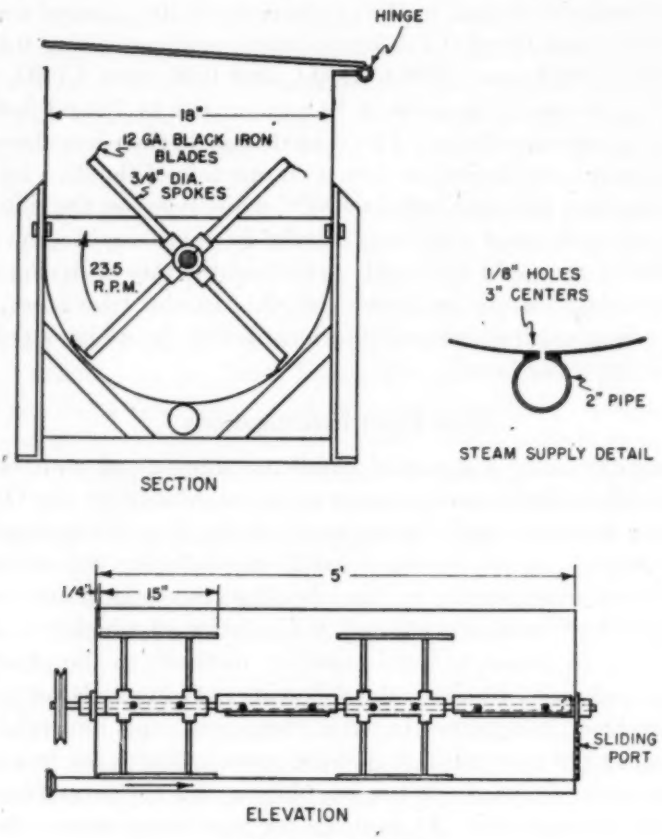

Fig. 1. Cooker detail.

which very badly interfere with mold growth, were eliminated. Hence, a pilot plant cooker was designed employing cooking with steam at atmospheric pressure. This cooker is shown in Figure 1. It consisted of a covered mixer, 18 inches in diameter by 5 feet long. The mixer was nondirectional, had a speed of 23 rpm, and produced efficient agitation in the bran mass. Steam was admitted through a row of jets at the bottom of the cooker. The cooker was of black iron, and no

serious corrosion occurred during operation over a period of several months.

The cooker was operated on a batch system. The bran, in 50-pound lots, was mixed with the acid and steamed in the covered cooker with continuous agitation for 30 minutes. Obviously the amount of dilution due to steam condensation in any installation of this kind would depend upon local conditions of steam quality. Experience with the pilot plant installation showed that use of 0.2 *N* hydrochloric acid, containing double the desired concentration of mineral salts, mixed in the ratio of two parts bran to one part acid resulted in the desired final moisture content of about 51%. Plating tests on the cooked bran showed no growth of foreign microorganisms after incubation for several days. Cooling was accomplished by means of an air stream blowing through the unit, with mixer running, until the temperature dropped to about 35°C. The spore inoculum was then added to the bran in the cooker by means of an insecticide duster, and the cooker was emptied by elevating one end, with mixer running, allowing the expulsion of the inoculated bran from a port in the end of the cooker, the inoculated bran being conveyed in baskets to the incubation units.

2. Inoculum Development. The development of an adequate method for producing spore cultures on the laboratory scale has been described above. The laboratory work demonstrated that the amount of bran which can be employed in a container is limited, thin layers being necessary if extensive sporulation is to occur throughout the mass. Moreover, the humidity and air supply must be carefully controlled to achieve most rapid growth, sporulation, and correct rate of drying. A method was still required for producing large amounts of spore inoculum for commercial operation. To accomplish this purpose, galvanized iron pans were constructed, 24 by 35 by 4 inches, provided with covers as shown in Figure 2. The pan cover had an air inlet at the center and air outlets, extending to within an inch of the bottom, at each corner.

For preparation of spore cultures in the pan, 4.5 pounds of the standard corn-bran-acid mixture is spread in it to a depth of about ½ inch, and the pan with contents is sterilized by heating with steam. The cooled medium is inoculated with mold spores and the pan is placed in a constant temperature cabinet at 32°C; it is desirable to have good air circulation around it. Air at 30° to 32°C is added through the center opening at the rate of 1200 to 1800 ml per minute. At the end of the 4th or 5th day, when heavy sporulation has been obtained, the cover is removed and the pan is placed in the cabinet

again. The spore culture dries within 24 hours and is then ready for use.

3. Methods for Inoculation. The method developed for preparing spore inoculum was fully satisfactory for large scale use, but in a large mold bran plant, if 1% inoculum were employed as in the laboratory, the requirement of inoculum per day would necessitate the use of so much spore culture that the inoculum development unit would be of unwieldy size. Two lines of investigation were followed in finding a solution for the inoculation problem. First the value of mycelium

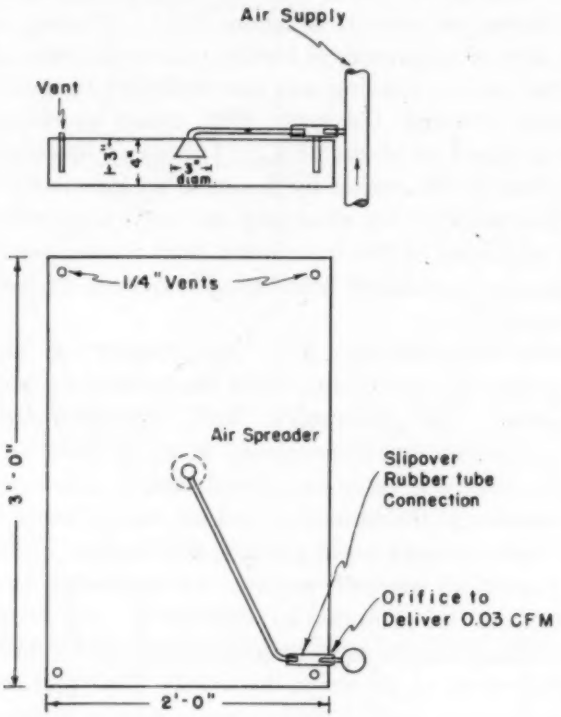

Fig. 2. Inoculum pan.

transfer was studied, and it was found that two mycelium transfers could be used before contamination with other molds, spores of which survived the sterilization, became very serious. However, this scheme for inoculation was inconvenient and a study was next made to determine the influence of the inoculation ratio using spores. No evidence was at hand that the 1% ratio used in the laboratory was either optimum or minimum. To obtain satisfactory spore distribution, an insecticide duster was used to transfer the spores to the bran in a mixer which continually exposed fresh bran to the inoculum, various

ratios of spore inoculation being tried. It was found that even as low an inoculation ratio as 0.04% of dry spore culture was satisfactory, and mold growth was as rapid and as heavy as with higher inoculation ratios. Lower inoculation ratios than 0.04% resulted in slower and less prolific mold growth. It was finally concluded that spore inoculation was definitely superior and more convenient than mycelium inoculation, and, to allow for possible lower spore viability, it was decided to employ 0.10% of dry spore inoculum in all subsequent pilot plant investigations.

4. Methods of Incubation. Investigation of methods for incubation of the growing mold and evaluation of the products produced occupied most of the time during the pilot plant research. After trying many testing methods and finding them wanting, the tedious fermentation procedure was used for the final evaluation of the mold brans prepared during the course of this work. To insure comparable results the same sample of wheat was used in all tests. This wheat was one of the lowest alcohol yielders tested in the Nebraska laboratories. Its relative alcohol-yielding capacity in comparison with other grain samples, expressed in terms of grams of ethanol produced per 100 g of total dry matter and employing the regular test method with optimum proportions of malt, was as follows: test wheat, 30.5; official No. 2 hard wheat, 33.5; official No. 2 spring wheat, 32.8; official corn, 36.0.

The details of the fermentation test method were as follows: In each 2-quart square form Mason jar 250 g ground wheat plus 2.5 g malt were mixed with 500 ml water at 63°C, held for 10 minutes at this temperature, and then heated in a water bath at such rate as to raise the temperature to 90°C in 30 minutes. The mashes were then heated in the autoclave for one hour at 15 pounds steam pressure. The jars were removed, one at a time, from the autoclave and the mash allowed to cool to 90°C. Then the requisite amount of mold bran (or malt when this was under test) as a slurry in 600 ml of cold water (15°C) was added quickly with rapid stirring. The jar was then placed in a cold water bath, and when the mash temperature reached 32°C it was inoculated with 33 ml of a culture of yeast, No. 567 of the Northern Regional Research Laboratory collection, grown in potato wort. The fermentations were incubated for 72 hours at 32°C, and then analyzed for alcohol. The alcohol yields were based upon loss of weight during incubation and alcohol recovered by distillation.

The laboratory research had shown that when the bran contains the proper moisture and nutrients, and is inoculated at 32° to 35°C with good spore inoculum, the spores germinate in about 3 to 4 hours and the temperature of the bran starts rising in about 5 to 6 hours,

assuming there is sufficient oxygen available for the metabolic processes of the mold. Aeration must be started at that time and it is highly desirable to avoid mechanical disturbance of the bran from that point until the bran particles are well covered, because the young mold mycelium is tender and easily damaged. Oxidative reactions continue at an increased tempo, reaching a peak at about the 15th hour, then declining during the next 15 to 20 hours. Aeration may be employed to maintain proper temperature control; and, to avoid serious drying during this operation, the air should be nearly saturated with moisture.

Incubation of the mold on bran in trays with natural aeration was investigated, using four horizontal trays, as shown in Figure 3, to

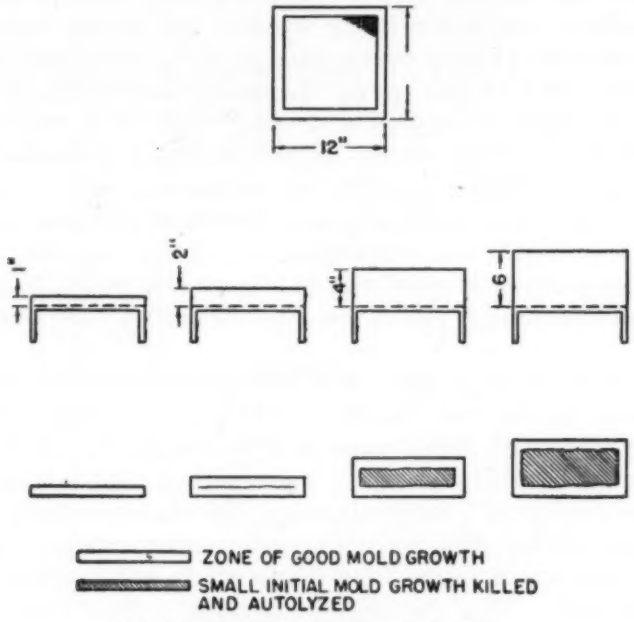

Fig. 3. Tray incubation with natural aeration.

permit study of the influence of layer thickness. These trays were placed in a humidified cabinet after being filled with inoculated bran from the pilot plant cooker, so that natural circulation of air around and through the bran layer was not impeded. The maximum layer depth at which uniformly good mold growth was obtained was 2 inches. With greater bran layer depths, there was a layer of good mold growth about 1 inch thick on the outside, and inside this the temperature reached such high levels that the small initial growth was killed and autolyzed.

The principal virtue of tray incubation is its simplicity. The disadvantage is the tremendous tray area required. In a mold bran unit

for processing one ton of bran per day, provision for 36 hours incubation on the trays would mean a tray requirement for 3000 pounds of bran. Assuming 15 pounds of dry bran per cubic foot (some brans give weights as low as 13 pounds per cubic foot) and a 2-inch bran depth, a tray area of 1200 square feet is required. Assuming trays 6 by 6 feet, there would be required 34 trays, which for this size unit would mean emptying and refilling one tray per hour. This is not unreasonable, but probably a minimum size of commercial plant would expect to process at least 10 tons of bran per day, which would mean about 12,000 square feet of tray area and the handling of about 10 trays per hour if the trays were 6 by 6 feet in size. The mechanical operation of handling so many trays thus becomes unwieldy. Such large tray areas present a handling problem which was not solved during the period of pilot plant work.

The method of incubating the growing mold on thick layers of bran with aeration under pressure was investigated exhaustively. In order to obtain data on the influence of the temperature of incubation and of the moisture content of the bran, it was necessary to develop a small incubation cell by trial and error procedure. After a number of failures, a wooden cell 12 by 12 by 12 inches was built with a false bottom and top, each with a screened air inlet or outlet 4 by 4 inches with a 4-inch unperforated border, and equipped so that air flow could be easily reversed. This cell had a capacity of 10 pounds of dry bran at a 10-inch depth and the false top was designed to fit snugly in the box so that it could be pressed down to rest on top of the bran. Temperatures in various parts of the bran were measured with thermometers inserted through the side of the cell. The air was saturated with moisture at the desired temperature, and pressure was measured by means of open tube manometers. The air pressure was changed by throttling as necessary to maintain a volume of air that would hold the temperature within the desired range and the direction of air flow was reversed at hourly intervals. As the rate of oxidation increased and as the interstices between the bran particles became filled with the mold mycelium, increased air pressures were required to maintain the desired temperatures. A record of the air pressure was kept and it was assumed that since this is a good measure of heat evolution, it is also a good measure of amount and rate of mold growth, up to a point where the amount of mold growth is such as to decrease markedly the permeability of the bran.

It was soon found that temperature of incubation and moisture content of the bran are dependent variables, and a number of runs at several moisture contents had to be made at each temperature of incubation. The influence of the temperature with moisture content

optimal at each level, selected from the numerous runs, is shown in Figure 4. It was concluded that with proper adjustment of moisture, the most rapid mold growth is obtained between 32° and 38°C. At this temperature the best moisture content was secured when 8 parts of 0.1 *N* hydrochloric acid were employed with 10 parts of bran by weight. The moisture content of the bran used was 12%, hence the moisture content of the wet bran as it was placed in the incubator was

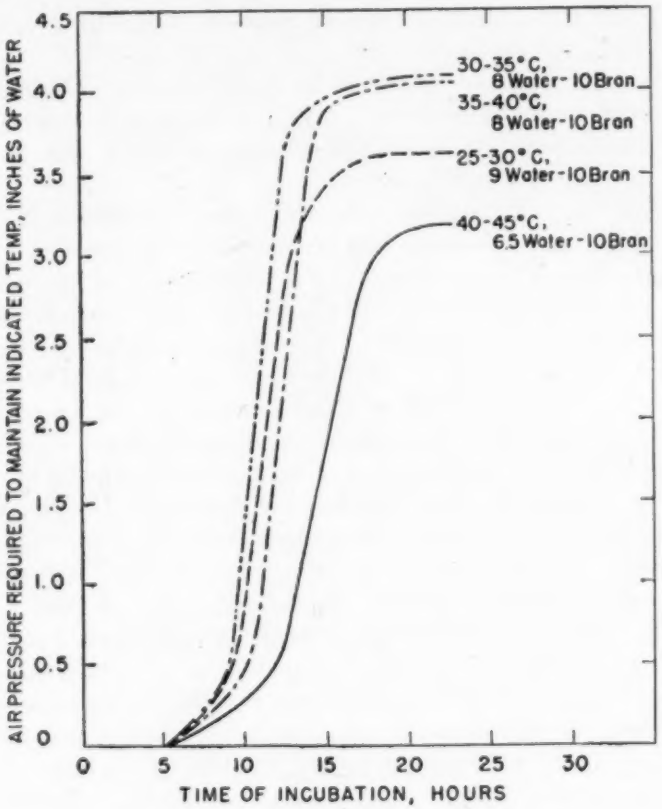

Fig. 4. Influence of temperature upon rate of mold growth.

51%. In all subsequent pilot plant experiments using pressure aeration these conditions of moisture content and temperature were adhered to.

The small horizontal cell gave good mold bran under optimum conditions of temperature and pressure, and duplicate runs gave products which were remarkably uniform as measured by fermentation tests. The effect on the quality of mold bran produced in the horizontal cell when incubated for various times under optimum conditions of

temperature (32° to 38°C) and air pressure to maintain the proper temperature is shown by the data of Table I. It is obvious that, within the limits of experimental error, alcohol yields obtained with the mold bran produced in the small horizontal pilot plant cell on incubation for 36 hours were equivalent to the yields obtained with an excellent laboratory preparation, although somewhat more mold bran was required to obtain the maximum alcohol yield. The data also show that incubation for periods of less than 36 hours resulted in mold bran which had lower saccharifying ability.

TABLE I

TIME OF MOLD INCUBATION AND ALCOHOL YIELD

Mold bran, %	Ethanol yield, g/100 g total dry matter					
	Mold bran incubated for					Laboratory mold bran
	20 hrs	24 hrs	28 hrs	32 hrs	36 hrs	
0.2	—	—	—	—	—	23.1
0.5	—	—	—	—	—	26.5
0.7	25.5	25.9	27.5	26.0	27.8	—
1.0	26.6	27.3	29.0	28.0	29.5	29.8
1.5	28.1	29.0	29.8	29.0	30.8	30.9
2.0	29.0	29.6	30.3	29.8	31.0	31.5
2.5	29.9	30.5	30.7	30.5	31.1	—
3.0	30.4	30.7	30.9	31.0	31.4	31.6
4.0	30.6	31.2	31.1	31.2	31.4	—

Since the small horizontal cell gave satisfactory mold bran when operated under optimum conditions, it was considered possible that a similar cell of greater size might be employed for larger scale work. However, a larger horizontal cell has several marked disadvantages, the most important being difficulty in emptying.

Another cell arrangement is vertical, which would provide for ease in filling at the top of the cell and for emptying at the bottom. A vertical cylindrical cell proved a complete failure and a vertical cell rectangular in shape was designed with vertical hardware cloth faces through which air under pressure could be forced through the bran in the cell. A modified form of such a cell is shown in Figure 5. Much of the pilot plant work was done with a cell of this type, having a bran cell 3 by 3 by 1 feet in size, with 8-mesh hardware cloth faces and air spaces on each face of the cell. To prevent loss of air around the bran in the cell, an unperforated border was used. This closed the air path for that portion of bran within the borders, but there is a certain amount of diffusion which serves these areas. Obviously there is a limit to the width of these borders. By trial and error method, it was

found that 4 inches was the practical limit of the border, and this width was used in constructing the vertical cell. The first run showed that the bran packed badly at the bottom, and a vertical cell could probably not be more than 3 feet high because of this packing. The best design was found by constricting the bran compartment near the top so that as the bran settled it filled the constricted section and thus reduced the air leakage over the top of the bran, and this modified cell is the one shown in Figure 5.

Later a similar type of incubation cell, also with 9 cubic foot capacity, was constructed, in which the bran cell was inclined at an angle of 45° as shown in Figure 6. This cell was as easy to fill and empty as the vertical cell, and packing was not serious.

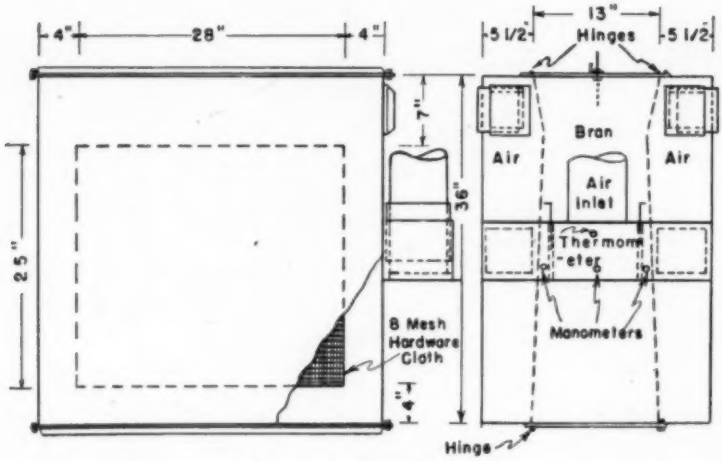

Fig. 5. Vertical incubation cell.

The allowable bran layer thickness is limited by two factors, air pressure required and temperature difference through the layer. Of course the temperature gradient may be changed by changing the air flow, so the two are dependent variables. The maximum temperature difference through a 12-inch layer at the peak of heating, with the rates of aeration described in this paper, was about 10°C, which is as large as can be tolerated without definite reduction in rate of mold growth. All of the pilot plant cells provided for 12-inch bran layers.

In order to determine accurately the air pressure and volume required, measurements were made using the two types of 9 cubic foot cells described. In Figure 7 are shown graphs of typical data. The incubation temperature was held within the optimum range, 32° to 38°C, and the moisture content was initially about 51%. It was found that the best control was obtained when the inlet air tempera-

ture was 30° to 32°C and the air was 85 to 90% saturated with moisture. The exhaust air temperature reached 36°C at the peak of heating, and at several points in the cell the bran temperature was 38°C.

From graphical analysis of the air volume curve of Figure 7, it may be estimated that the air requirement for 36 hours of incubation averages 32 cubic feet per minute per cubic foot of bran, with a maximum

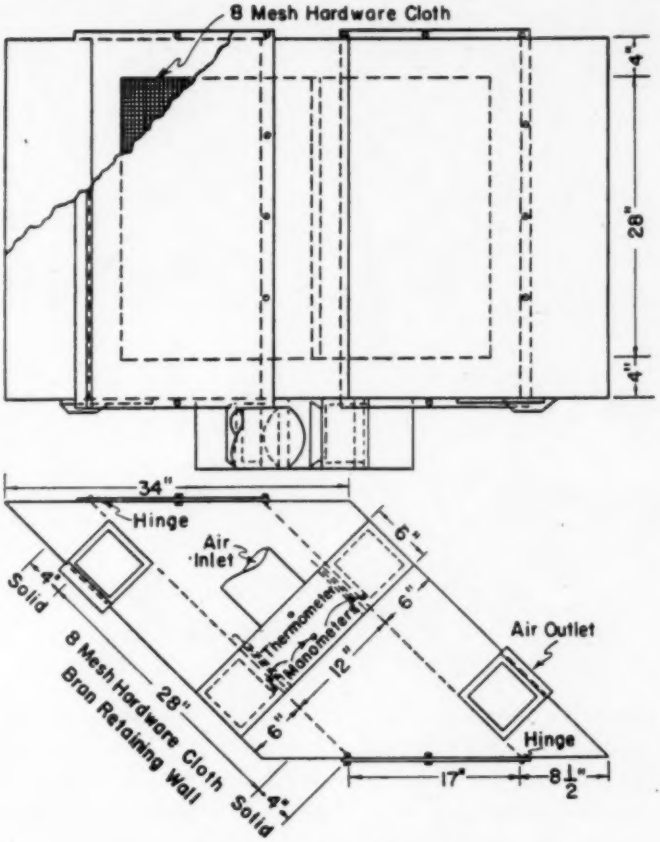

Fig. 6. Inclined incubation cell.

at the peak heating period of 52 cubic feet per minute. These measurements include the air lost by reason of inevitable small leakage in ducts and at the cell.

In the laboratory method for preparing mold bran by incubating in a pan with forced aeration, the bran is removed from the pan shortly after the peak heating period, is broken into small pieces, and held with reduced natural aeration another 24 hours, during which there occurs marked increase in mycelial growth. To investigate the effect of a

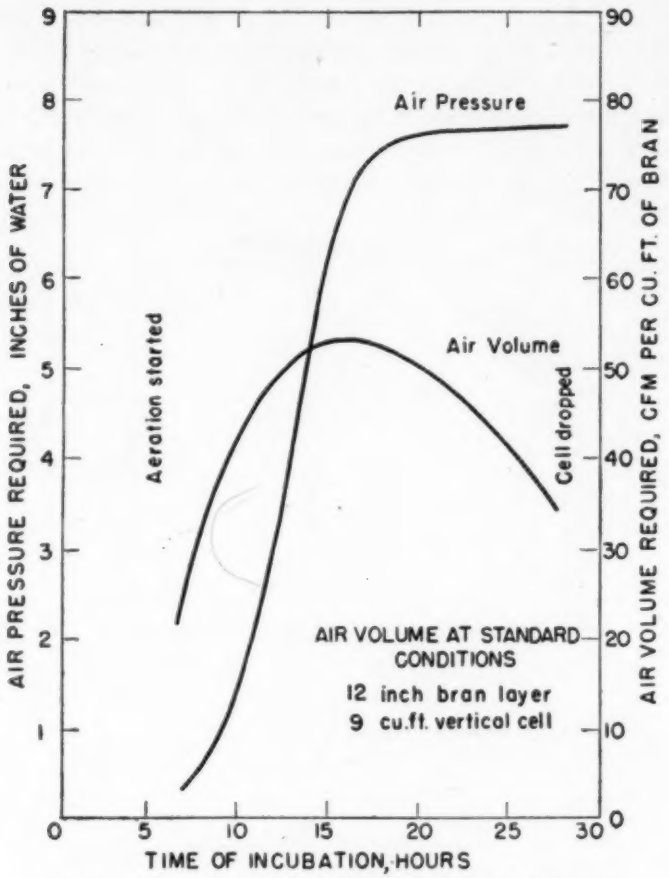

Fig. 7. Air pressure and volume requirements during mold growth.

similar two-stage incubation system on a larger scale, samples of mold bran were taken from a pilot plant cell after 18 hours incubation, were broken up and then incubated under laboratory conditions to produce very heavy mycelial growth. These were tested by the standard fermentation procedure, yielding the data of Table II. The extra mycelial growth obtained by using the two-stage incubation gave a

TABLE II
MOLD BRANS WITH HEAVY AND LIGHT MYCELIAL GROWTH—
36 HOURS TOTAL INCUBATION

Mold bran %	Ethanol yield, g/100 g dry matter	
	Light growth, single stage incubation	Heavy growth, two stage incubation
1.5	27.3	30.0
3.0	30.9	31.9

higher alcohol yield, and less mold bran was required. The data indicate that there is a certain amylase production accompanying the large mycelial production during the secondary incubation stage, although the desired amylases are produced largely during the early stages of growth. The desired amylases are largely extracellular, and in the mold bran must be primarily present in the whole bran mass and not just in the mold mycelium.

Secondary incubation on the pilot plant scale confirmed these results. For pilot plant operations it was necessary to take the mold bran from the primary cell incubator at a point after maximum heat production, which occurs at about the 15th hour, break it into pieces one inch diameter or smaller, and hold it loosely packed in the secondary incubator about 18 to 20 hours. To obtain data on the air pressure and volume requirement for the secondary incubator, a run was made in which the bran was cooked, cooled, and inoculated in the usual manner, and was then incubated in a 9 cubic foot cell for 18 hours at 30° to 35°C. It was then removed from the primary incubator, broken into pieces, and placed in four secondary incubators, consisting of compartments with perforated bottoms, in 1-, 2-, 3-, and 4-foot depths. Air pressure and volume required to maintain the temperature at 26° to 38°C were measured during the 18 hours of secondary incubation.

It was barely possible to maintain temperature control in the 3-foot layer, while the 4-foot layer got completely out of hand. For commercial operation it appeared that a 2- to 3-foot layer should be employed. It was found permissible to use a lower air temperature during this second incubation stage than in the primary, good results being obtained when the incoming humidified air was between 26° and 30°C. The air requirement for the 2-foot depth was 22 cubic feet per minute per cubic foot (12 pounds dry bran) at 6 inches water pressure.

The pilot plant work employing vertical and inclined cells showed that it was feasible to produce mold bran in incubators providing forced aeration through layers of bran, and a design for a commercial plant was developed, employing blocks of inclined cells 6 by 6 by 1 feet in dimensions for primary incubation, with a drag conveyor, or bins with perforated bottoms for secondary incubation.

5. Usage and Storage. During the pilot plant work the mold bran was generally dried so that it could be stored and used as needed, because it was convenient to make a number of mold bran products before testing them. It was found that the material containing 25 to 30% moisture as it came from the incubation cells could be dried rapidly because of the large surface exposed, and in the pilot plant the wet product was simply put in bins with false bottoms through which

dry air was blown. The bran layers could be as thick as 4 feet, but it was indicated that the temperature of the air should not exceed about 45°C or there would be a serious loss of amylase. Drying has little effect either upon the amount of mold bran required or upon the alcohol yield obtained. Hence, if mold bran is made in an alcohol plant where it may be used as produced, the recommended practice would be to make up the moist mold bran into a slurry with cold water for use in the plant. This slurry should not be held for more than an hour or two, in the interest of reducing the chance for development of contamination. The concentration of the slurry would, of course, be adjusted to the particular plant requirements. If the mold bran were produced in a separate plant, it would be necessary to dry with a current of warm air, using any suitable type of dryer, to a moisture content below 15%, preferably about 12%. In this dry condition, if protected from outside moisture, the material may be stored in bags or bins for long periods without deterioration.

Semicommercial and Commercial Production of Mold Bran

A semicommercial mold bran unit, with a capacity of about one ton of mold bran per day, patterned after the system developed by the pilot plant research was designed and constructed in the alcohol plant of the Farm Crops Processing Corporation at Omaha. The cooker was similar to that of the pilot plant except that it was 6 feet by 24 inches. Inclined cells were constructed, in a battery, for the primary incubation unit, all with 6 by 6 foot screen dimensions. One of the cells, for experimental purposes, was 8 inches and one was 14 inches between screen faces. Three of the cells were 10 inches, and three were 12 inches between screen faces. As secondary incubators, closed bins with perforated metal bottoms were employed. Separate fans and humidifiers supplied air to the primary and to the secondary incubators.

The primary incubation cells worked fairly well when air control was carefully maintained, but the necessary regulation of air pressure was found to be very critical and temperature control was extremely difficult. Another major difficulty was the shrinkage of the bran, and it was necessary after incubation for a few hours to pack additional inoculated bran into the cells to prevent air leakage over the top of the mold bran "chunks." The secondary incubation was satisfactory. With careful control this semicommercial unit produced mold bran of fair quality, but by no means equal to standard laboratory preparations. The conclusion was reached that it would be unwise to attempt the construction of a larger plant to this design.

As previously mentioned, the simplest incubation system involves the use of trays with a bran depth of not over 2 inches. Shortly after

the semicommercial unit began production, a system for handling the requisite large tray area was conceived, and a new incubator installation was built, incorporating this method. The flow sheet for the operation of this plant, as drawn by Van Lanen and Blom of the Northern Regional Research Laboratory of the U.S.D.A., is shown in Figure 8. In this system the trays were made of standard 1½ inch iron pipe and approximately 5 by 14 feet in size. The trays were hinged lengthwise so that the front could be dropped for unloading and raised for filling, thus eliminating the labor incident to handling individual trays. The trays were hung in an incubation room built

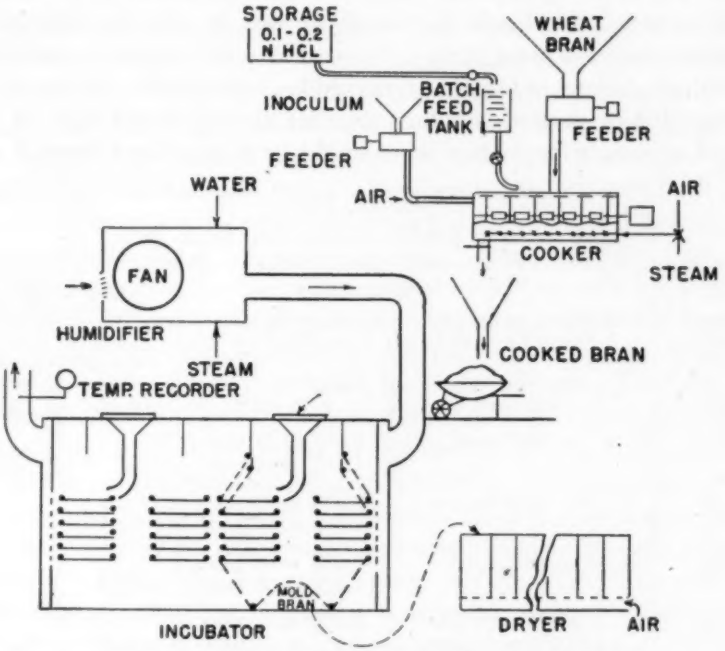

Fig. 8. Flow sheet for semicommercial mold bran production.

of tile and provided with air ducts for circulating humidified air. The same cooker as for the previous installation was employed, and the secondary incubator of the original installation was used, with dry air, for drying the mold bran. Control was simple with this installation and mold growth was excellent, the product being very uniform in quality from day to day, and of high amylolytic activity.

Both the cell unit and the tray unit were operated for a period of about 9 months at the Omaha plant, most of the time simultaneously, in order to produce the quantities of mold bran required for testing its use on the plant scale. These tests were so favorable that one of the government's idle hemp-processing plants, at Eagle Grove, Iowa, was converted to produce mold bran at the rate of 10 tons per day.

Aside from certain mechanical difficulties attendant on the operation of a new plant, the only major difficulty experienced at the Eagle Grove plant has been difficulty in procuring bran. This has resulted in great variability in the bran processed, necessitating frequent changes in the operating conditions as regards moisture, nutrient requirements, and the like. However, commercial operation has demonstrated the soundness and feasibility of the process for the commercial production of mold bran.

Commercial Uses of Mold Bran

The use contemplated for the mold bran produced at the time of the erection of the Eagle Grove plant was in the manufacture of industrial alcohol from grains. The commercial tests with mold bran in the alcohol plant at Omaha, reported by Underkofler, Severson, and Goering (1946), showed conclusively that alcohol yields were at least as good or somewhat better when mold bran was used instead of or

TABLE III

RESULTS OF PLANT-SCALE TESTS OF MOLD BRAN

Number of fermenters	Saccharifying agent in fermenter mashes	Saccharifying agent in yeast culture mashes	Average alcohol yield per standard bushel	
			Proof gal	Gal 190 proof
299	10% malt	22% malt	4.77	2.51
847	9 to 10% malt	8.6% malt plus 4.3% mold bran	5.17	2.72
6	4% mold bran	8.6% malt plus 4.3% mold bran	5.24	2.76
12	9 to 10% malt	8.6% malt plus 4.3% mold bran	5.15	2.71
7	3.9–6.2% malt plus 2.2–0.9% mold bran	8.6% malt plus 4.3% mold bran	5.26	2.77
12	9 to 10% malt	8.6% malt plus 4.3% mold bran	5.23	2.75

along with malt for saccharifying the fermentation mashes, and saccharification was accomplished at less cost. Typical plant yield data, taken from the paper of Underkofler, Severson, and Goering (1946), are shown in Table III. Research has indicated also that mold bran may find use in other fermentation processes as a source of enzymes and/or growth factors.

Recently the sugar shortage has resulted in the development of a tremendous demand for enzyme-converted grain syrups, and pilot plant studies were made on the use of mold bran as the converting agent for preparing such syrups. These experiments showed that

better results were obtained when combinations of mold bran and malt were employed than when either was used alone for the saccharification. Such mold bran-malt combinations are now in use for the large-scale commercial production of enzyme-converted grain syrup.

Expanded use of mold bran in other industrial fields using enzymes, such as in production of adhesives, paper sizing, sizing and desizing of textiles, and the like, may be expected, but most probably these industries will employ enzyme concentrates from the mold bran rather than the crude product.

Summary

Laboratory methods for growing the mold *A. oryzae* on moist, sterilized wheat bran for the production of a low-cost enzyme product, mold bran, have involved growth in a rotating drum, growth on trays in specially humidified and ventilated incubators, and growth in thick layers in a covered pan having a perforated bottom for forced aeration through the bran mass. Thorough sterilization of the moist bran, which can best be effected by using dilute acid and heating with steam, and a period of quiescence during the early growth phases have been found essential for the production of mold bran of highest amylolytic power, and the pan method of incubation was preferred for laboratory work. An efficient method for the production of laboratory spore cultures to serve as inoculum has also been developed.

Pilot plant investigations led to the solution of the main problems in translating laboratory results to commercial production. A cooker was developed for the sterilization, involving a covered mixer with direct steam injection. The sterilized bran was also cooled in the mixer by means of an air stream, and was inoculated in the mixer by blowing dry spore culture into the mixer. It was found that mycelium inoculation was possible, but spore inoculation was preferable, a ratio as small as 0.04% of dry spore culture giving as good results as higher inoculum ratios. Spore cultures for inoculum were best prepared on a large scale by the use of covered pans through which was circulated just enough air to provide for the metabolic requirements of the mold.

Pilot plant investigations of methods of incubation involved growth on trays and in cells with forced aeration. The tray method was simpler, but no satisfactory method of handling the large tray area required for commercial operation was devised during the pilot plant research. A pilot plant method involving the use of either vertical or inclined cells having hardware cloth faces so that air could be forced through the bran was developed. With an initial moisture content of about 51%, most rapid mold growth occurred between 32° and 38°C. The temperature was controlled by varying the rate of air flow through the bran layer by increasing the pressure as required. Two-stage

incubation gave somewhat better mold bran than incubation for the entire period in a single cell.

Semicommercial production of mold bran in two units showed that a unit involving incubation on swinging trays in a specially ventilated room was easier to operate and gave a more uniform product than a unit designed in accordance with the pilot plant method using cells. Optimum conditions of moisture content, nutrient requirements, temperature range, air requirements, incubation time, and the like, ascertained during the pilot plant research with cells hold equally for the process using the swinging trays. A commercial plant processing 10 tons of bran per day is now in successful operation.

Large-scale uses for mold bran have developed in saccharifying grain and potato mashes for the alcoholic fermentation and in saccharifying grain for the production of enzyme-converted syrups.

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GRAIN STORAGE STUDIES. V. CHEMICAL AND MICROBIOLOGICAL STUDIES ON "SICK" WHEAT [1,2]

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Among the most poorly defined types of damage in wheat associated with storage deterioration is the condition known to the grain trade as "sick" wheat. This damaged condition is manifested by kernels showing a dull appearance with discolored or blackened embryos. Mold growth is usually present on commercial samples of such grain, its viability is low, and the fat acidity is high.

Swanson (1934) found that mold growth on wheat could be inhibited by the exclusion of air from the containers or by treatment of the seeds with Ceresan (ethyl mercury phosphate), and yet sick wheat would develop with time in samples so stored. He also noted that under such conditions, serious damage to baking qualities did take place in the absence of mold growth, but this deleterious effect did not show itself in increased fat acidity.

Thomas (1937), on the other hand, believed sick wheat to be due to toxic principles elaborated by molds, principally the species *Aspergillus flavus*, which grow on wheat and reduce its viability when the moisture content is sufficiently high.

The most comprehensive investigation of this phenomenon is that of Carter and Young (1945) who produced sick wheat in the laboratory by storing Fulcaster wheat in sealed containers at various moisture contents and temperatures over time intervals up to 687 days and followed the changes in fat acidity and the decrease in germination. They arbitrarily eliminated all samples on which molds were visible and concluded that sick wheat formation is associated with anaerobic storage. In general an increase in sick kernels, favored by high moistures and temperatures, was accompanied by an increase in fat acidity and a decrease in germination.

The present study was undertaken to (1) determine the various types of microflora occurring on sick wheat, (2) determine the influence of temperature, moisture, time, and storage atmosphere on the de-

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velopment of sick wheat and associated microfloral contaminants, and (3) follow these changes by indices of chemical deterioration.

Methods

Quantitative determination of the number of sick or germ-damaged seeds in various samples was made by the Federal Grain Inspection Office, Minneapolis, Minnesota. Assay was made of the number of seeds showing internal microfloral infection, as well as the types present, by a technique involving surface disinfection with sodium hypochlorite and plating the seeds on Smith-Humfeld agar (Smith and Humfeld, 1930). Germination of various wheat samples was determined by the State Seed Testing Laboratory, University Farm, St. Paul, Minnesota, except for special studies for which germination tests on wet filter paper were made.

The technique used for experimental milling of wheat samples, as well as methods of determination of moisture by the two-stage air-oven method, fat acidity, and sugars, is outlined in *Cereal Laboratory Methods* (4th ed., 1941).

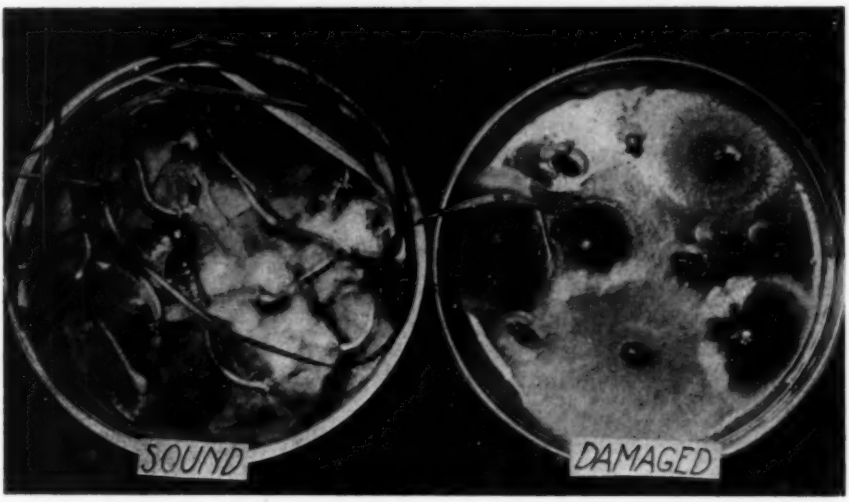

Fig. 1. Sound and germ-damaged wheat from the same lot on Smith-Humfeld agar 10 days after surface disinfection. Most of the sound seeds have germinated; the molds growing from them are Alternaria, Fusarium, and Helminthosporium. None of the germ-damaged seeds have germinated; species of Aspergillus and Penicillium as well as bacteria are growing from these seeds.

Germination and Microflora of Sound and Sick Wheat

Small samples of healthy and germ-damaged seed were hand picked by the Federal Grain Inspection Office, Minneapolis, Minnesota, from each of 12 carloads of wheat received from various points in Montana, North Dakota, South Dakota, and Minnesota in February and March,

1945. All of the lots were graded either musty or sour. Fifty seeds of the sound and germ-damaged samples were surface disinfected and placed on Smith-Humfeld agar in petri dishes. Figure 1 is a photograph of typical sound and germ-damaged seed, 10 days after this treatment. Figure 2 is a close-up view of a germ-damaged seed from

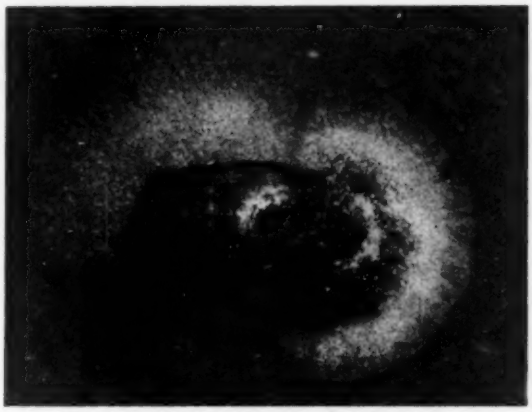

Fig. 2. Close-up view of a germ-damaged wheat seed seven days after surface disinfection (from the same lot as shown in Figure 1, right).

the same lot as shown in Figure 1, right. The percentage of germination and number and kind of molds that grew from each seed are recorded in Table I.

The molds inhabiting the sound seeds, namely, the species of Alternaria, Fusarium, and Helminthosporium, were those commonly present in sound wheat in this region at the time of harvest. None of these are able to grow at a seed moisture content below 25%, and evidence presented elsewhere in this paper indicates that they disap-

TABLE I

PERCENTAGE OF GERMINATION AND INTERNAL MOLD INFECTION OF SOUND AND GERM-DAMAGED WHEAT OBTAINED FROM COMMERCIAL SOURCES [1]

Kind of wheat	Germination	Mold infection	Kinds of molds and percentage of each in the total population
	%	%	
Sound	86.7	64.5	Alternaria 90%, Fusarium 5%, Helminthosporium 5%
Germ-damaged or "sick"	1.4	76.0	*Aspergillus glaucus* 60%, Penicillium 20%. Remaining 20% made up of *A. niger*, *A. flavus*, *A. candidus*, Nigrospora, Rhizopus, Trichoderma, and several unidentified bacteria

[1] Average of 12 samples.

pear relatively rapidly from seed stored at 18% moisture. Even at higher moisture contents they appear unable to compete successfully with other molds, notably the Aspergillus species, which grew out from the seed. The majority of the molds isolated from the germ-damaged seed, on the other hand, make up the dominant flora which proliferate at moisture contents between 14.5 and 20%, *Aspergillus glaucus* being able to grow on seeds with moisture contents below 16%, *A. candidus* at 17%, *A. flavus*, certain Penicillia, and possibly certain bacteria at from 18 to 20%. Some of the minor molds that were present in the germ-damaged seed, such as Nigrospora, Trichoderma, and Rhizopus, do not grow on wheat until a moisture value between 20 to 25% is reached, and their presence in a few of the seeds suggests that the moisture content of at least some individual seeds in these samples had exceeded 20% at some time during storage.

Effect on Viability of Inoculation of Sound Wheat with Microorganisms Isolated from Sick Wheat

In studies of the effect of inoculation of sound wheat with microorganisms found in sick wheat on seed viability, at 18% moisture, a sample of irrigation-grown hard red spring Montana wheat (hybrid variety), which was found to be virtually free from internal molds, was used. Portions of the internally mold-free wheat were placed in 8-ounce screw-capped medicine bottles and a 0.5% aqueous solution of sodium hypochlorite was added to eliminate microorganisms from the outside of the seed as well as to bring the moisture content up to 15%. After 24 hours, sterile water in which mold spores or bacteria were suspended was added to bring the moisture content up to 18%. Three molds, namely *A. candidus*, *A. glaucus*, and *A. ochraceus*, and four unidentified bacteria isolated from sick wheat were tested. Three controls were used, all of which received the preliminary surface disinfection described above when the moisture content was raised to 15%. To one, only sterile water was added to raise the moisture content from 15 to 18%; the moisture content of the second was raised from 15 to 18% with a 1% solution of sodium hypochlorite; the third was identically treated as the first, but after the water was absorbed it was dusted with 0.2 g chloramine B per 100 g of seed. Three replicate bottles of each control and of each inoculated sample were used. The bottles were stored at room temperature (22° to 26°C) and samples were removed after 38, 111, and 201 days, surface disinfected, and placed on sterile moist filter paper to determine the germination. The results are summarized in Table II. It should be emphasized that the methods of surface disinfection used did not eliminate all the microorganisms in the seed of the controls.

TABLE II

VIABILITY OF MONTANA WHEAT INOCULATED WITH VARIOUS ORGANISMS AND STORED AT A MOISTURE CONTENT OF 18% FOR VARIOUS LENGTHS OF TIME

Treatment	Percentage of germination after		
	38 days	111 days	201 days
Control, 0.5% sodium hypochlorite	93	49	0
Control, 1.0% sodium hypochlorite	91	67	0
Control, 0.5% sodium hypochlorite plus dusting with Chloramine B (0.2 g/100 g)	90	67	0
Bacterium No. 1	92	42	0
Bacterium No. 2	95	29	0
Bacterium No. 3	84	36	0
Bacterium No. 4	91	19	0
Aspergillus candidus	36	14	0
Aspergillus glaucus	74	35	0
Aspergillus ochraceus	34	24	0

The three molds were tested again on the Montana wheat, but this time at 20% moisture after storage for 22 days, germination being determined on agar and in nonsterile soil. The viability of the inoculated and noninoculated seeds is given in Table III.

In both tests *A. candidus* and *A. ochraceus* reduced the viability of seeds rather rapidly, while *A. glaucus* reduced the viability somewhat more slowly. These molds produce heavy tufts of sporophores on the germ ends of the seeds in six to eight days at moisture contents of 16 to 20%, although later sporophores may arise from the entire surface of the seeds. Typical seeds stored at 16% moisture for 10 days are illustrated in Figure 3. In both tests, most of the dead seeds had the dark germ which is typical of sick wheat.

The role of bacterial growth in the loss of viability of these stored samples is less clear than that of the molds. The multiplication of bacteria on and in the seed is less easy to follow by macroscopic and microscopic examination than is the case with the molds. In fact,

TABLE III

VIABILITY OF MONTANA WHEAT INOCULATED WITH THREE DIFFERENT MOLDS AND STORED FOR 22 DAYS AT 20% MOISTURE

Treatment	Percentage germination after 22 days	
	On agar	In nonsterile soil
Control, surface disinfected	100	82
Inoculated with:		
Aspergillus candidus	48	29
Aspergillus glaucus	76	71
Aspergillus ochraceus	54	52

no external evidence of bacterial proliferation was evident on seeds stored at these moisture values. The results of Table II do, however, suggest that Bacterium number 4 and possibly numbers 2 and 3 contributed to the deterioration of wheat stored at 18% moisture, and tests now under way indicate that these bacteria seem to multiply slowly on or in autoclaved and subsequently inoculated wheat held at a moisture content of 18%. Further evidence on this point will appear in a later portion of this paper.

Fig. 3. Sound, surface-disinfected wheat seeds stored for 10 days at 16% moisture. Tufts of *Aspergillus glaucus* sporophores are growing out of them, chiefly from the germ end.

Influence of Moisture, Temperature, Time, and Interseed Atmosphere on the Development of Sick Wheat and Contaminating Microflora

To secure data on the influence of various environmental factors on the development of sick wheat, an experiment was carried out in which wheat was stored at two moisture and temperature levels under different gaseous atmospheres. For this study a lot of sound Regent seed wheat, grown at McIntosh, South Dakota, in 1944, showing 94% germination was used. Two lots of wheat were conditioned to 12.1 and 18.0% moisture, and 500-g portions of each lot were distributed among a number of ordinary quart-size mason jars. The regular flat metal covers were perforated and one-inch lengths of $\frac{3}{16}$-inch copper tubing were soldered to these openings. After sealing with the metal screw ring, closure was effected by means of a short length of neoprene tubing and a screw clamp. Each lot of every moisture level was divided into three groups of equal number. Atmospheres of carbon dioxide, nitrogen, and oxygen were then introduced by evacuating the air in the jars with a vacuum pump followed by introduction of the commercial compressed gases. This procedure was repeated for each jar to ensure virtually complete displacement of the air by the gas. The various jars were then stored for six months at two temperatures: room temperature (22° to 26°C) and 5°C. The grain stored at room

temperature was sampled and assayed for germination, sick kernels, fat acidity, and sugars at monthly intervals. Samples at 5°C were examined at three-month intervals. Extensive microfloral examination was carried out on the samples stored at room temperature at the end of two and five months.

After two months' storage, samples of the gaseous atmosphere in the jars containing nitrogen and oxygen, which were stored at room temperature, were withdrawn and analyzed for carbon dioxide using the Haldane-Henderson gas analyzer. A high concentration of this gas was found and this necessitated the reflushing of the balance of the oxygen and nitrogen jars of this moisture group at monthly intervals with fresh gas. The concentration of carbon dioxide after monthly periods is shown in Table IV.

TABLE IV

CONCENTRATION OF CARBON DIOXIDE APPEARING IN JARS CONTAINING REGENT WHEAT STORED AT 12.1 AND 18.0% MOISTURE UNDER INITIAL ATMOSPHERES OF NITROGEN AND OXYGEN

Time of sampling	Storage temperature	Carbon dioxide in interseed atmosphere			
		Moisture 12.1%		Moisture 18.0%	
		Nitrogen atmosphere	Oxygen atmosphere	Nitrogen atmosphere	Oxygen atmosphere
Months		%	%	%	%
2	Room	0.8	0.9	35.1	92.4
3	Room	0.5	13.9	21.2	97.6
4	Room	0.6	0.4	8.3	79.9
5	Room	0.5	0.7	5.2	88.1
3	5°C	0.1	0.1	5.2	4.9

Rather rapid respiratory exchange of oxygen to produce carbon dioxide can apparently occur in sealed containers in one month of storage at 18% moisture. Even under anaerobic conditions (nitrogen atmosphere) considerable carbon dioxide production occurs. Had the jars contained only normal air (20.9% oxygen), oxygen depletion would be expected to occur even more rapidly.

Data for fat acidity, reducing, nonreducing, and total sugars, germination, and percentage of sick or germ-damaged kernels in the various samples are given in Table V. Results of the microbiological examination at the end of the second and fifth months of storage obtained by surface-disinfecting 50 seeds of each lot with sodium hypochlorite, plating on sterile Smith-Humfeld agar, and incubating at room temperature for 10 days are shown in Table VI.

Chemical deterioration, both as regards lipolysis and sugar hydrolysis, was more rapid in the samples at 18% moisture than at 12.1%

TABLE V

INFLUENCE OF MOISTURE CONTENT, TEMPERATURE, TIME, AND INTERSEED ATMOSPHERE ON THE CHANGES IN CHEMICAL COMPOSITION, GERMINATION, AND FORMATION OF SICK KERNELS IN REGENT WHEAT

Time of sampling	Storage temperature	Initial moisture 12.1%			Initial moisture 18.0%		
		CO_2	N_2	O_2	CO_2	N_2	O_2
Months	FAT ACIDITY, MG KOH PER 100 G DRY WHEAT						
0	Room	13.8	13.8	13.8	13.8	13.8	13.8
1	Room	17.8	19.7	23.0	18.8	20.2	25.7
2	Room	24.1	24.1	22.6	44.3	22.7	31.3
3	Room	36.2	35.3	35.2	40.3	39.1	54.1
4	Room	24.1	23.2	23.3	26.3	25.2	46.3
5	Room	19.2	19.3	19.3	23.4	22.7	34.7
6	Room	23.2	23.3	23.3	29.4	31.6	40.9
3	5°C	35.3	30.4	32.8	39.6	44.6	43.3
6	5°C	21.7	21.7	21.1	21.1	20.5	24.2
	REDUCING SUGARS AS MALTOSE MG PER 10 G						
0	Room	51	51	51	51	51	51
1	Room	46	51	44	56	54	54
2	Room	40	44	45	60	66	58
3	Room	40	31	32	57	58	55
4	Room	49	49	49	109	101	87
5	Room	46	48	49	119	119	94
6	Room	44	44	43	116	126	93
3	5°C	28	31	32	45	39	35
6	5°C	41	41	43	48	50	49
	NONREDUCING SUGARS AS SUCROSE MG PER 10 G						
0	Room	221	221	221	221	221	221
1	Room	207	214	214	202	193	187
2	Room	210	216	213	169	169	167
3	Room	176	143	143	107	117	101
4	Room	230	226	218	138	128	119
5	Room	222	227	242	124	118	119
6	Room	218	208	220	114	126	106
3	5°C	127	143	135	170	150	129
6	5°C	214	217	216	215	196	199
	TOTAL SUGARS, MG PER 10 G						
0	Room	272	272	272	272	272	272
1	Room	253	265	258	258	247	241
2	Room	250	260	258	229	235	225
3	Room	216	174	175	164	175	156
4	Room	279	275	267	247	229	206
5	Room	268	275	291	243	237	213
6	Room	262	252	263	230	252	199
3	5°C	155	174	167	215	189	164
6	5°C	255	258	259	263	246	248

TABLE V—*Continued*

Time of sampling	Storage temperature	Initial moisture 12.1%			Initial moisture 18.0%		
		CO_2	N_2	O_2	CO_2	N_2	O_2
Month		REDUCING SUGARS AS % TOTAL, %					
0	Room	18.8	18.8	18.8	18.8	18.8	18.8
1	Room	18.2	19.2	17.1	21.7	21.9	22.4
2	Room	16.0	16.9	17.4	26.2	28.1	25.8
3	Room	18.5	17.8	18.3	34.8	33.1	35.3
4	Room	17.6	17.8	18.4	44.1	44.1	42.2
5	Room	17.2	17.5	16.8	49.0	50.0	44.1
6	Room	16.8	17.5	16.3	50.4	50.0	46.7
3	5°C	18.1	17.8	19.2	20.9	20.6	21.3
6	5°C	16.1	15.9	16.6	18.3	20.3	19.8
		GERMINATION, %					
0	Room	94	94	94	94	94	94
1	Room	94	96	94	92	95	86
2	Room	97	98	97	76	69	63
3	Room	93	94	91	5	20	17
4	Room	93	95	91	0	0	0
5	Room	92	90	94	0	0	0
6	Room	96	94	90	0	0	0
3	5°C	90	91	91	93	92	94
6	5°C	97	97	97	93	92	90
		SICK WHEAT, %					
0	Room	0	0	0	0	0	0
1	Room	0	0	1	4	2	7
2	Room	0	0	0	1.5	2	6.5
3	Room	0	0	0	25	30	70
4	Room	0	0	0	100	100	100
3	5°C	0	0	0	3	3	1.5

moisture. In addition, it is significant that the wheat stored under oxygen at the high moisture value showed considerably higher fat acidity values as the experiment progressed than did the samples at the same moisture value stored under carbon dioxide or nitrogen. Nevertheless, the development of sick kernels proceeded under the three atmospheres, all being 100% sick at the end of four months. Data for the third month, however, suggest that samples under oxygen tend to become sick somewhat more rapidly than in the other atmospheres. Total sugars showed a somewhat greater decrease in the 18% moisture sample stored under oxygen than was found in the other gases. The reason for these differences is indicated in Table VI; only under the oxygen atmosphere at 18% moisture did significant increases in

Aspergillus glaucus and *A. candidus* occur. These fungi are vigorous respiratory and lipolytic agents and, being strictly aerobic in their respiratory requirements, proliferate only in an atmosphere containing oxygen. It therefore seems that the biochemical activity of molds is additive, or synergistic with, other agencies which may be responsible for the formation of sick wheat. Thus, under natural conditions of storage, their deteriorative action would enhance the rate at which sick wheat develops. These fungi occurred on all samples of naturally formed sick wheat.

TABLE VI

EFFECT OF TIME ON INTERNAL MICROFLORAL POPULATION OF REGENT WHEAT STORED AT 12.1 AND 18.0% MOISTURE UNDER ATMOSPHERES OF CARBON DIOXIDE, NITROGEN, AND OXYGEN AT ROOM TEMPERATURE

Time of sampling	Moisture at sampling	Atmosphere	Percentage of seeds internally infected						
			Total	Bacteria	*A. glaucus*	*A. candidus*	*A. flavus*	Alternaria	Other fungi
months	%								
2	12.1	CO_2	32	—	—	—	—	30	2 (unknown)
	11.9	N_2	32	—	—	—	—	32	—
	11.6	O_2	30	—	—	—	—	30	—
	17.8	CO_2	0	—	—	—	—	—	—
	18.0	N_2	4	—	—	—	—	2	2 (unknown)
	18.0	O_2	2	—	—	—	—	2	—
5	12.6	CO_2	10	—	—	—	—	10	—
	12.2	N_2	24	—	—	—	—	24	—
	12.4	O_2	18	—	—	—	—	18	6 (Penicillia)
	18.4	CO_2	82	82	2	—	—	—	—
	18.4	N_2	68	68	—	8	—	—	—
	18.5	O_2	100	90	50	24	2	—	—

The fact that a large proportion of the sick seeds showed bacterial contamination at the end of five months which was not apparent after two months of storage suggests that either the proliferation and biochemical activity of the microflora may be responsible for the sick condition, or that the progressive loss of viability and increasing chemical deterioration of the seeds due to other factors make it possible for the bacterial inoculum initially present to grow more readily. The literature on the minimal moisture requirements of microflora, which has been reviewed by Milner and Geddes (1946), would suggest that bacterial proliferation is not to be expected at relative humidities below 95%. The equilibrium relative humidity for wheat at 18% moisture is approximately 86%, according to the data of Coleman and Fellows (1925) and Rozsa (1935).

As previously mentioned, the common seed contaminant Alternaria rapidly disappears in the presence of moisture conditions favorable to the proliferation of other species such as the Aspergilli.

Distribution of Fat Acidity in the Milled Fractions of Sound and Sick Wheat

After six months, the remaining wheat samples stored at room temperature were experimentally milled. As a control, a sample of the original dry Regent wheat which had been stored at approximately 5°C in contact with a normal air atmosphere was milled. The various milling fractions were then analyzed for fat acidity with the results shown in Table VII.

The difference between the distribution of fat acidity in the various fractions of the normal or nonsick wheats in comparison with those from the sick wheats is very striking. The control sample, and those stored at 12.1% moisture under carbon dioxide, nitrogen, or oxygen, in which no sick wheat developed, shows the highest concentration of fat acidity in the bran fraction; the acidity decreases regularly in the order of mill fractions removed by successive stages of refinement, i.e., bran, brown shorts, white shorts, low grade, clear, and patent. In contrast, the fat acidity distribution in the milled fractions of sick wheat increases from the bran (which, except for the sample stored under oxygen and on which mold growth occurred, had a value comparable to the normal wheat brans) to a high maximum peak in the low grade flour fraction, followed by a regular decrease in the clear and patent flour. It is to be noted that while the milling yield of similar fractions from the various samples differed somewhat, the variation was not of a magnitude such as to affect the identity of the fraction. Hence the corresponding fractions may be considered to have approximately the same fat content, and thus the fat acidities can be logically compared.

While the total fat content of the various fractions was not determined, due to an insufficient sample, the considerable information in the literature on the distribution of total fat among commercial mill fractions of comparable wheats (Sullivan *et al.*, 1927, 1928, 1940, and Barton-Wright, 1938) shows that total ethyl or petroleum ether extract falls regularly with increasing refinement, and that the fat content of the low-grade flour fraction is usually only little more than one-half of that of bran. The fat acidity values in the normal or nonsick wheats analyzed appear to parallel these expected total fat contents rather closely.

Discussion

These studies are primarily of an exploratory nature but they indicate that a number of complex variables are probably involved in the formation of sick wheat. They indicate that the deterioration is not solely a result of the proliferation and metabolic activity of molds such as *Aspergillus glaucus*, *A. flavus*, and *A. candidus* which are

TABLE VII

FAT ACIDITY OF MILLED FRACTIONS OF REGENT WHEAT STORED FOR SIX MONTHS AT ROOM TEMPERATURE AT 12.1 AND 18.0% MOISTURE UNDER ATMOSPHERES OF CARBON DIOXIDE, NITROGEN, AND OXYGEN

Storage moisture	Storage atmosphere	Sick kernels	Fraction	Milling yield of fraction	Fat acidity[1] (air-dry basis)
%		%		%	
Control	Air (5°C)	0	Bran	8.7	69.4
			Brown shorts	5.9	61.2
			White shorts	6.4	41.1
			Low grade	0.8	42.9
			Clear flour	1.3	33.3
			Patent	76.9	20.1
12.1	Carbon dioxide	0	Bran	10.1	74.9
			Brown shorts	4.8	69.9
			White shorts	6.1	49.3
			Low grade	0.9	46.6
			Clear flour	1.9	33.3
			Patent	76.2	22.4
12.1	Nitrogen	0	Bran	9.8	70.3
			Brown shorts	6.3	64.4
			White shorts	5.8	45.7
			Low grade	0.9	44.3
			Clear	2.0	32.4
			Patent	75.2	21.0
12.1	Oxygen	0	Bran	11.4	63.9
			Brown shorts	4.7	55.7
			White shorts	5.5	45.7
			Low grade	0.9	42.9
			Clear	1.8	32.4
			Patent	75.7	21.5
18.0	Carbon dioxide	100	Bran	9.7	73.1
			Brown shorts	5.1	79.0
			White shorts	4.6	89.6
			Low grade	1.0	92.7
			Clear	1.8	41.6
			Patent	77.8	24.8
18.0	Nitrogen	100	Bran	11.3	73.1
			Brown shorts	4.1	77.2
			White shorts	4.7	95.9
			Low grade	1.0	127.0
			Clear	1.3	49.3
			Patent	77.6	21.0
18.0	Oxygen	100	Bran	8.3	93.1
			Brown shorts	6.3	115.6
			White shorts	4.8	199.1
			Low grade	0.8	257.1
			Clear	1.8	188.6
			Patent	78.0	39.2

[1] Mg KOH per 100 g.

indigenous to normal wheat, since wheat stored at high moisture under carbon dioxide and nitrogen where mold growth was inhibited became sick to the same extent as did that under an oxygen atmosphere where these molds proliferated. The deteriorative effect of mold growth is manifested principally in lipolytic activity and appears to be additive to other deteriorative processes responsible for sick wheat. In this regard, the conclusion of Carter and Young (1945) that the development of sick wheat may occur in an anaerobic storage atmosphere seems to receive confirmation. Certainly the inference of Thomas (1937) that sick wheat is primarily due to the metabolic activity of molds is not supported by the evidence in the present studies. However, the deteriorative effect of molds usually present under natural conditions of storage enhances sick wheat formation.

The fact that the respiratory activity of wheat stored in sealed containers at room temperature caused almost quantitative exchange of oxygen for carbon dioxide over relatively short intervals indicates that atmospheres must be controlled if experimental results are to be related to natural or commercial storage conditions. Milner and Geddes (1945) have shown that in a commercial bin, zones of heating in stored soybeans, which are centers of high respiratory activity, contain air which is nearly normal in oxygen content owing probably to convection effects at the heating centers. The gaseous composition of the atmosphere surrounding stored seeds is therefore of critical importance in determining the course of their deterioration.

The data presented in Table V suggest that bacteria are involved in the sick wheat phenomenon, as the viability of seeds treated with them is reduced more than control samples at the same moisture value. However, the moisture value at which this occurred (18%) is considerably greater than the lowest moisture level (12.2%) at which Carter and Young (1945) noted sick wheat formation after 279 days of storage at 40°C. It seems very unlikely that bacteria could be involved in the deterioration at such a low moisture value. As previously indicated, several workers who have classified microflora on the basis of minimal humidity requirements for growth agree that bacterial proliferation is not to be expected at relative humidity values below 95%. Nevertheless, the present data obtained with bacterial inoculation suggest that attention will need to be given to the possibility of bacterial growth at humidity levels lower than the data of these workers have shown.

The probability that the basic cause of the sick wheat condition, manifested by darkening of the embryo and eventually of the whole kernel, may be due to the inherent seed enzymes cannot be dismissed. Such enzymic activity may lead initially to a weakening of the germina-

tive powers and eventually to sufficiently extensive chemical deterioration to favor the proliferation of bacteria initially present only as a contaminating inoculum. That the growth of microflora on seeds is enhanced by germinative and chemical deterioration of seeds is apparent from the data of Milner and Geddes (1945a) who showed that soybean seed damaged by storage at a temperature (50°C) which allowed survival of mold spores yielded respiratory values at 30°C greatly in excess of those given by the same seed and contaminating molds stored at 30°C prior to the heat treatment.

The data of Johnson and Hagborg (1944) are pertinent in connection with the darkening which develops on sick wheat kernels. They found that at high temperature, especially when combined with a high humidity, melanistic areas may develop on the glumes, lemmas, peduncles, and internodes of Apex and Renown wheat in the absence of any infection by pathogenic organisms. The data of Johnson and Hagborg suggest that enzymic or even such nonbiological effects as interaction between carbohydrates and nitrogen-containing compounds (Maillard or "browning" phenomenon) are possible under such conditions.

That the darkening of sick wheat, which appears first in the embryo portion where high enzymic activity is to be expected, is probably not related to the increase in fat acidity is indicated by the data of Table VII. The fact that of the various mill fractions of sick wheat, the low-grade flour showed the highest fat acidity value, whereas the bran of normal wheats was highest in this factor in comparison to all other fractions, suggests that the lipolytic agencies in sick wheat do not occur primarily in the bran or in the germ (which accompanies the bran and brown shorts fractions in milling, and which has the highest fat content of any of the wheat tissues), but more probably in the aleurone and scutellar tissues. These tissues show a more organized active cellular structure than do other wheat tissues, and are credited with enzymic functions which become highly active on germination of the seed. The present results are directly in accord with the work of Sullivan and Howe (1933) who investigated the lipase activity of various milling fractions of normal wheat and found it to be concentrated in greatest amount in the clear and low-grade flour fractions. That fungi beneath the bran coat are probably not responsible for the lipolytic activity in sick wheat (except in the presence of oxygen as shown by this study) is suggested by the work of Oxley and Jones (1944) who found the mold contamination to be localized just inside the outermost bran tissue.

Further studies on the phenomenon of sick wheat will need to take into account the nature and distribution in the wheat tissues of various enzyme systems such as the lipases, esterases, and oxidizing enzymes

such as tyrosinase, as well as the moisture relationships which govern their activity. The exact role of bacteria in this phenomenon needs considerable clarification, and it would be of great advantage to grow wheat entirely free from contamination by molds and bacteria for such studies.

Summary

"Sick" or germ-damaged wheat samples showed very low germination values and were found to be infected principally with *Aspergillus glaucus*, *Penicillium* spp., and to a lesser extent by *A. candidus*, *A. flavus*, *A. niger*, and a few bacteria. In contrast, sound wheat samples from the same lots of grain were largely contaminated with Alternaria which disappeared when stored under moisture conditions favorable to the proliferation of the Aspergilli.

Surface-disinfected, relatively mold-free Montana wheat inoculated with various molds and bacteria isolated from sick wheat and stored in air lost viability faster than did noninoculated controls, and most of the nonviable seeds had symptoms typical of sick wheat. Molds were present on the noninoculated controls before the test was completed. The influence of bacteria was not so clearly defined as was the effect of the molds.

Sick wheat was produced in the laboratory by storing Regent wheat at 18% moisture under atmospheres of carbon dioxide, nitrogen, or oxygen, in sealed containers. Only under oxygen did molds, principally *Aspergillus glaucus* and *A. candidus*, proliferate throughout the period of the test, whereas sick kernels appeared under all atmospheres. Fat acidity increased in all samples at 18% moisture, but was greatest in the samples stored under oxygen. The sick wheat condition, therefore, was not entirely due to molds but their metabolic activity enhanced sick wheat formation.

Aerobic and anaerobic production of carbon dioxide by molds and seeds at 18% moisture in the sealed containers required renewal of the oxygen and nitrogen atmospheres at monthly intervals.

Fat acidity of mill fractions of sound wheat was at a maximum in the bran fraction, and decreased regularly toward the patent flour, more or less in the order of the total fat content of the various fractions. The milled fractions of sick wheat, on the other hand, showed highest fat acidity in the low-grade flour. The high lipase activity of the aleurone layer and scutellum, relative to that of the germ, is apparently responsible for this effect.

More information is needed on the location, distribution, and activity of inherent wheat enzymes, such as the lipases and oxidizing enzymes, to explain the formation of fat acidity and the darkening of sick wheat.

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REPORT OF THE 1945–46 COMMITTEE OF THE NEW YORK SECTION ON PROCEDURES FOR THE EXAMINATION OF FLOUR FOR EXTRANEOUS MATERIALS

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The food industry is continually confronted with new problems in sanitation. As more information is obtained, the problems are recognized and, in general, conscientious efforts are made to attain improvements. Sanitary precautions in handling and preparing food products pay dividends by the improvement of quality, elimination of waste, and protection of the consumer. To insure a high standard of quality, the analyst is called upon to develop practical methods and techniques for the detection of sources of contamination, which may be present but are not detectable by macroscopic examinations.

The executive committee of the New York section of the American Association of Cereal Chemists realized the need to familiarize the industrial analyst with procedures for the examination of food products for extraneous materials. A committee of chemists was appointed in November, 1945, to make a study of procedures available, and, if possible, to recommend those most adaptable to the needs of the average industrial analyst. The importance of simplicity and rapidity in the procedures was recognized, since in most industrial laboratories engaged in ingredient control, the analyst must be prepared to handle a large number of samples daily.

In order to make the procedures practical, it was deemed necessary to condense instructions and simplify manipulations as much as possible and still have a reasonable assurance that, when followed by the analyst, a separation of most of the extraneous materials would be obtained.

As the committee was composed of cereal chemists, procedures for the examination of flour were given paramount consideration. Various procedures were suggested and collaborative examinations of samples were made. The clearest fields which contained the greatest amount of extraneous materials were obtained by making gasoline and mineral oil flotation separations from charges digested with pancreatin and hydrochloric acid.

The training of the analyst and his ability to identify extraneous materials must always be taken into consideration; but with the aid of instructions which have been published recently by the Food and Drug

Administration [1] and the Association of Official Agricultural Chemists,[2] the average industrial analyst should be able to follow these suggested procedures for the examination of flour and obtain a reasonably accurate evaluation of the amount and kind of extraneous materials which it contains.

Two methods of digestion of the flour were studied: one by pancreatin, the other with hydrochloric acid. Gasoline and light mineral oil were the two flotation media used. The pancreatin digestion gasoline flotation procedure is especially applicable to whole wheat and graham flours. The mineral oil flotation tends to trap considerable bran and thereby produce fields which are difficult to examine. With white flours, the hydrochloric acid digestion and mineral oil flotation procedure is rapid and simple in manipulation. Pancreatin digestion and gasoline flotation can also be applied to white flour if preferred, and if the over-all time required to obtain results is not too important.

Description of essential apparatus, a list of required reagents, pertinent information, and the procedures follow:

Examination of Flours for Extraneous Materials

APPARATUS

(a) Sieve—10 mesh U. S. Standard.

(b) Special separatory funnel (Figure 1)—1 liter capacity Squibb pear-shaped without ground-in glass stopcock. The lower end should shape down evenly and seal onto a glass stem approximately 3″ long with a bore of approximately $\frac{5}{16}$″. A piece of rubber tubing is fitted over the stem and a pinch clamp used for releasing and closing the funnel. A rubber stopper is most satisfactory for stoppering the top of the funnel.

(c) Wildman trap flask (Figure 2)—1–2 liter Erlenmeyer flask into which is inserted a close-fitting rubber stopper supported on a stiff brass rod of approximately $\frac{3}{16}$″ diameter and 2–3″ longer than the height of the flask. Rod is threaded at lower end and fitted with nuts and washers to hold the stopper tightly in place. Lower nut must be countersunk into the rubber to prevent striking the flask. All metal fittings should be brass or a metal which does not corrode readily in salt solutions or in slightly acid or alkaline solutions.

(d) Filtration funnels and accessories—Büchner or Hirsch funnels for filtration with suction. Funnel should be of such diameter that filter papers will fit closely against the sides. A close

[1] Microanalysis of Food and Drug Products. Food and Drug Circular No. 1, 1944.
[2] *Official and Tentative Methods of Analysis*, Chapter 42, 6th ed., 1945.

fitting disc of bolting cloth placed between the perforated funnel plate and filter paper accelerates filtration and when properly fitted helps prevent possible loss of material around the edges of the paper. Filter papers should be rapid acting. They should be ruled with parallel lines at such a distance apart that the field from line to line is visible under the microscope at the magnification at which the examination is being made. Paper ruled with parallel lines 7 mm apart (such as 7 mm ruled Shark Skin supplied by Schleicher & Schüll Co.,

Fig. 1. Special separatory funnel.

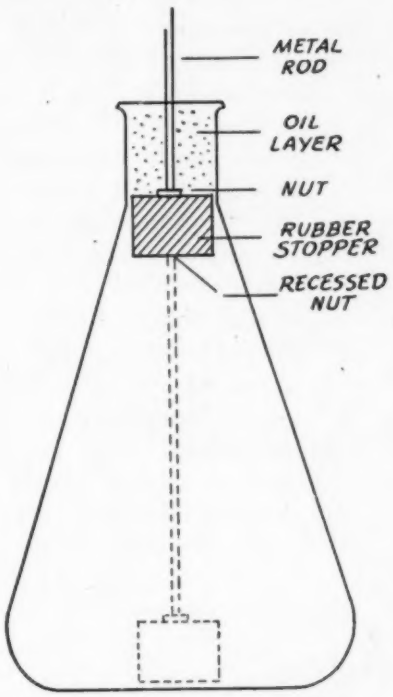

Fig. 2. Wildman trap flask.

N. Y.) is satisfactory when making examinations at magnifications up to 30 diameters. When making examinations at magnifications of 30–40 diameters it is more convenient to have the lines closer together. Lines ruled with oil, alcohol, and waterproof ink are most satisfactory, but for ordinary work a suitable paper can be provided by ruling a smooth-surfaced qualitative filter paper with a sharp-pointed medium-hard black lead pencil. Filter papers of 12.5 cm diameter are most suitable for use where considerable material is liable to be carried onto the fields.

(e) Plates for holding filter papers for microscopic examinations—A convenient plate can be made by cementing a square piece of light plate glass approximately 3″ × 3″ onto a piece of thin, smooth asbestos board of the same dimensions.

(f) Wet pads and covers—Convenient pad for keeping filter papers wet until examinations are made can be provided by placing a smoothly folded towel on a table or glass plate and saturating with water. Filter papers to be examined should be placed on a wet pad and covered with a suitable cover to prevent dirt from settling upon them.

(g) Microscope—Greenough-type binocular microscopes which afford reasonably wide fields and magnifications of 20–40 diameters for making routine examinations, and higher magnifications of 75–100 diameters to aid in making identifications of small objects, are most convenient for use in examining fields such as those obtained when flotation separations are made from flour and other food products.

(h) Illuminator—The most suitable light is provided by a low-voltage bulb set in an adjustable mount in an elliptical reflector so that light of suitable intensity can be focused directly into the field from one direction. The light should be mounted on a jointed arm to permit focusing from all angles. A low-voltage lamp provided with adjustable transformer permits the adjustment of light intensity to the requirements of the observer and the work. The intensity of the light must be changed frequently depending upon the color of the objects being investigated, color of the background, and the magnification at which the examination is being made.

(i) Dissecting needles.

REAGENTS

All water and reagents should be filtered or examined to insure that they contain no extraneous materials.

(a) Pancreatin U.S.P.

(b) Hydrochloric acid. Concentrated C.P.

(c) Gasoline. Maximum boiling point 130°C.

(d) Mineral oil. Light grade, white petroleum or paraffin oil.

(e) Alcohol. Ethyl or isopropyl may be used for washing field or breaking emulsions where required.

(f) Oil solvents. Benzol, petroleum ether, or gasoline may be required at times to aid in dissolving the mineral oil and washing it from the equipment and filter paper.

GENERAL INFORMATION

Flour from which small particles of extraneous materials are to be recovered should be thoroughly mixed with water and the viscosity reduced to a minimum by heating and digesting with pancreatin or dilute hydrochloric acid.

Gentle stirring is advised to aid in loosening insect fragments, hairs, etc., but violent agitation which may disintegrate fragments and filth particles should be avoided. Violent shaking of digested mixtures with gasoline or oil may also cause emulsions which will hold large amounts of flour and carry it onto the paper.

Pancreatin seems to be the most suitable enzyme for digesting flour and cereal products as its action is fast on both carbohydrates and proteins. The optimum temperature for its action is 40°C. The optimum pH of pancreatic amylase is 6.7–7.2 and pancreatic trypsin 7.8. Unless the flour to be examined is chemically treated, it is not usually necessary to adjust the pH of the cooked water-flour mixture. Pancreatin has little or no effect on insect fragments or hairs.

Insect fragments, hairs, and the usual light particles of extraneous materials are not noticeably affected when boiled with concentrations of 2–3% hydrochloric acid for a reasonable length of time.

Experience is necessary in identifying insect parts, hairs, and other extraneous materials under the microscope. This experience can best be gained by examining known specimens at the magnifications and under the conditions that extraneous materials which are separated from food products are likely to be found.

Only fragments of filth which can be definitely identified should be reported as such. Comments as to doubtful materials should be made. A uniform type of report should be kept by the analyst which will give an adequate description of extraneous materials found, and the quantity of sample examined.

PROCEDURE

Section I. Preliminary Examination for Large Elements of Extraneous Materials. Examine closely around container seams and bag-ties, especially where covered, and where dust has accumulated. If the material is finely ground, smooth out a thin layer with a flour slick or some flat instrument and examine the surface carefully. If the product will pass a 10 mesh U. S. sieve, scoop up portions from the surface and around the edges of the container, and screen. Examine for larvae, insects, clumps of webbing, insect and rodent excreta pellets, and other extraneous materials.

Section II. Digestion of Sample and Separation of Light Extraneous Materials. Digestion of the flour sample may be accomplished by either pancreatin or hydrochloric acid.

Part A. Pancreatin Digestion. Weigh charge (28–50 g flour) and transfer it to a liter beaker. Add 400 ml water and mix until a smooth suspension free from lumps is obtained. Heat the suspension very carefully with continuous stirring over a gas burner on an asbestos-centered wire gauze until it boils, or in a boiling water bath with stirring until the temperature reaches 85°C and continue heating for about 15 minutes. Cool to 40°–45°C. Add 0.5–1.0 g pancreatin by sprinkling the powder over the surface and stirring it through the suspension. Wash the adhering paste from the sides of the beaker back into the suspension. Allow to digest at a temperature of 35°–45°C until the viscosity of the mixture is reduced to a minimum and the insoluble materials settle readily. This usually takes 3 to 6 hours. Digestions may continue for much longer periods of time without harmful effects on white flours. It is sometimes advantageous to allow digestion to continue overnight, but when digesting graham or whole wheat flours which contain much bran, more satisfactory flotation separations of the light extraneous materials can be obtained after digestions of only 2 to 3 hours. If the digestions continue for longer periods of time, the light bran is loosened and when the flotation separations are made, it is carried onto the fields to be examined along with the light extraneous materials. The bran blocks the fields and makes it difficult to find the insect fragments, hairs, etc.

The gasoline flotation separation of the digested mixture may be accomplished by means of a separatory funnel or a Wildman trap flask as described below.

USE OF THE SPECIAL SEPARATORY FUNNEL. The separatory funnel should be a long narrow type, with a short stem having a fairly large bore (Figure 1). This shape is preferred because coarse materials can be mixed with the gasoline or oil and drawn off while maintaining as much distance as possible between the oil-water interface and the outlet. A long heavy wire or thin glass rod may be used to dislodge coarse materials should the stem become clogged. If the mixture contains particles too coarse to pass through the funnel, the water mixture should be mixed thoroughly with the gasoline or oil in the beaker, and the liquid portion decanted into the funnel.

Transfer the mixture obtained by pancreatin digestion from the beaker to the separatory funnel and add enough water to bring the volume up to about 800 ml. Add 25–30 ml of gasoline (max. b.p. 130°C). Stopper the funnel and roll gently in a horizontal position for 3 to 4 minutes. Invert the funnel a few times and release the pressure by

slightly opening the pinch clamp. After thorough mixing to bring the gasoline into contact with all particles, place the funnel in a stand and allow the materials to separate and settle for 5 to 10 minutes, if making separations from white flour. When making separations from graham, whole wheat, and coarse flours, it is advisable to draw off the coarse materials into a large beaker as they settle and decant liquid portions back into the funnel until all the coarse materials which settle have been removed from the funnel. This prevents packing and closing the funnel and also aids in loosening small fragments and hairs which tend to adhere to the coarse particles of cereal.

After settling, draw off several 200-ml portions to within 3 to 4 inches of the gasoline-water interface and decant the liquid portions back into the funnel. Wash all materials down from the inside of the separatory funnel and wash two or three times with 50–100 ml portions of water. Transfer the materials at the interface to a lined filter paper to be described later.

The digested mixture and washings from the first separation should be saved for further flotation separations. For the usual routine examination, a second gasoline flotation separation is advisable. For very thorough work, a third and fourth flotation may be necessary, but unless the product is unusually heavily contaminated, two careful flotation separations should be sufficient.

Operation of the Wildman trap flask. If the Wildman trap flask (Figure 2) is used in making the gasoline flotation separations, transfer the digested water-flour mixture to the flask, or add the flour and water and carry out the digestion in the flask. Add 25–30 ml gasoline, when it is to be used as the flotation medium, and stir it thoroughly through the mixture. Care must be taken that a frothy emulsion is not created which will entrap much flour. Fill the flask with water until the oil layer is brought into the neck so that when the stopper is raised until it closes off the neck of the flask, it will be about 1 cm below the oil layer and floating materials. Stir the contents of the flask gently to loosen light extraneous materials and allow to settle for 10–15 minutes. Spin stopper carefully to remove adhering flour before raising it. Raise the stopper until it closes off the bottom of the neck of the flask and decant the trapped-off oil layer and adhering materials into a 250 ml beaker. Rinse any adhering materials from the rod and neck of the flask into the beaker with alcohol and then water. Another portion of gasoline should be added and the contents of the flask again stirred thoroughly so that any light materials which may have been caught during settling and adhering to the surface of the flask may be loosened and recovered. Add water to

bring the oil layer up into the neck of the flask, allow to settle, trap off, and wash as directed for the first separation.

Where mineral oil is to be used as the flotation medium a second separation should be made using gasoline. The gasoline does not adhere so readily to the rod and sides of the flask and tends to loosen the adhering materials better than the mineral oil.

If the materials trapped off into the beaker are noticeably emulsified, add about an equal volume of ethyl or isopropyl alcohol. The contents of the beaker are now ready to be filtered.

Part B. Acid Digestion. Weigh charge (28–100 g flour—as much as 200 g of patent flours may be used), and transfer to a liter beaker. Add 400 ml dilute hydrochloric acid solution (20 ml concentrated 36.5% HCl to 380 ml water), and stir until the flour is thoroughly mixed with the solution. Heat carefully until the mixture begins to boil. Add 20 ml light mineral oil and continue the boiling for 15–20 minutes. A stirring rod should be placed in the beaker to prevent the boiling mixture from bumping and spattering. Cool to room temperature and carry out the mineral oil flotation separation in either the separatory funnel or in the Wildman trap flask as already described.

All materials sticking to the beaker should be carefully rubbed loose and washed into the funnel or flask.

After separating the oil layer by either the separatory funnel or the flask it is ready for filtering.

Section III. Filtration and Examination. Draw the oil contents of the funnel or flask onto a filter paper 12.5 cm diameter which has previously been moistened and fitted into a filter funnel. Mild suction should be used in filtering and care should be taken to obtain an even, and not too dense, distribution of the materials over the paper to be examined. When making flotation separations on flour containing considerable bran and germ, some of the light fragments are often held at the oil-water interface and carried onto the filter paper. For this reason paper of 12.5 cm diameter is recommended and can be readily examined under a wide field binocular microscope when cut into quarter sections. If the oil and water holding the entrapped materials which are to be drawn onto the filter paper are noticeably emulsified, the addition of an equal volume of ethyl or isopropyl alcohol will usually break the emulsion and prevent it from clogging the filter. Mineral oil may be washed through the paper with an oil solvent.

Microscopic Examination of Filter Paper. Place the wet, lined filter paper containing the separated materials on a flat plate and examine by moving along in the field of vision under the microscope.

For making routine examinations 25–40 diameters should be used. Higher magnifications of 75–100 diameters are convenient and should be used in making identifications of small objects. The entire filter paper containing the separated materials should be examined

A fine sharp dissecting needle should be used to move particles around in the field of vision The appearance from various angles and the consistency will greatly aid the analyst in identifying particles of materials.

Results

Members of the committee and collaborators examined two samples each of white and whole wheat flours. The samples contained considerable amounts of insect fragments and a few rodent hairs of the size which would be found in a flour sample milled from contaminated wheat. After examining the first samples by a number of procedures, the committee decided that most satisfactory results were obtained by the pancreatin-gasoline and acid-mineral oil procedures essentially as described above. A second sample each of white and whole wheat flour was then examined following these procedures and also by procedure M2B, as given in Food and Drug Circular No. 1. Results of these examinations are listed in Table I.

Comparable results were obtained by both procedures on examination of white flour. The hydrochloric acid digestion–mineral oil flotation separation is the more rapid of the two procedures and probably the more suitable for most control work. Collaborative results showed that the pancreatin digestion–gasoline flotation separation procedure is more applicable to whole wheat flours. Too much bran and germ is carried onto the field by the hydrochloric acid digestion–mineral oil procedure.

Agreement among collaborators on the type of extraneous materials found was satisfactory. Actual counts varied considerably but in general the agreement between analysts who were experienced in making such examinations was satisfactory. A determination of extraneous materials by microscopic examination is not quantitative to the degree of a quantitative chemical determination. Factors such as nonuniformity of samples and the ability of the analyst to recognize small particles of extraneous materials make it almost impossible to obtain strict checks. Quantitative results, however, are not actually necessary to differentiate between a clean flour and a flour which is contaminated.

Summary

Two simplified procedures for the examination of wheat flours for extraneous materials are described. In one, the flour is digested with

TABLE I

COLLABORATORS' RESULTS FOR EXTRANEOUS MATERIALS IN FLOUR BY THREE METHODS (RESULTS REPORTED ON POUND BASIS)

Collaborator	Extraneous materials	White flour			Graham flour		
		Procedure			Procedure		
		M2B	Pancreatin—gasoline	Acid—mineral oil	M2B	Pancreatin—gasoline	Acid—mineral oil
1	Insect fragments	54	110	200	126	96	112
	Rodent hairs	0	9	5	9	0	0
2	Insect fragments	191	378	351	85[1]	279	—
	Rodent hairs	0	0	0	0	0	—
3	Insect fragments	—	656	390	Could not	976	517[1]
	Rodent hairs	—	0	9	make count[1]	0	0
4	Insect fragments	182	290	291	218[1]	309	127[1]
	Rodent hairs	0	4	0	9	9	9
5	Insect fragments	355	200	251	36	46	82
	Rodent hairs	4	0	0	4	0	0
6	Insect fragments	—	—	452	—	—	—
	Rodent hairs	—	—	18	—	—	—
7	Insect fragments	36	80	32	0[1]	0[1]	16
	Rodent hairs	0	0	16	0	0	0
8	Insect fragments	48	32	96	80	32	160
	Rodent hairs	0	0	0	0	0	16
9	Insect fragments	279	360	315	—	198	180
	Rodent hairs	0	0	0	—	0	0
10	Insect fragments	448	464	358	416	736	64[1]
	Rodent hairs	0	16	0	0	0	0
11	Insect fragments	—	486	855	No count[1]	315	No count[1]
	Rodent hairs	—	36	9		18	

[1] Collaborator reported that the field contained entirely too much bran and germ to be examined satisfactorily.

pancreatin solution after which the extraneous material is separated by flotation with gasoline employing a separatory funnel or a Wildman trap. In the other, the flour is digested with hydrochloric acid solution and a light mineral oil used for the flotation. The extraneous materials are collected on filter paper and examined microscopically.

In a collaborative study, both procedures gave comparable results with a sample of white flour; hydrochloric acid digestion and flotation with mineral oil was the more rapid. With a sample of whole wheat flour, digestion with pancreatin and flotation with gasoline was more

satisfactory than the hydrochloric acid–mineral oil procedure, as the mineral oil flotation tended to trap considerable bran and produce fields which were difficult to examine.

Although the actual counts reported by different collaborators varied considerably, agreement on the types of extraneous materials was satisfactory.

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SOME CHARACTERISTICS OF GLIADIN AND GLUTENIN INDICATED BY DISPERSION AND VISCOSITY[1]

MARK A. BARMORE [2]

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Since gluten has been shown by Finney (1943) to be the material responsible for the differences in loaf-volume-producing ability of hard wheats, it would appear that a more thorough knowledge of the composition and properties of gluten is essential to an understanding of this characteristic. The present paper reports some preliminary results in the separation and study of gluten components.

The extensive literature reviewed by Bailey (1944) on methods of obtaining protein fractions and studying their properties attests to the importance of the subject. Haugaard and Johnson (1930), Sandstedt and Blish (1933), McCalla and Rose (1935), Blish (1936), Spencer and McCalla (1938), and McCalla and Gralén (1940, 1942) divided gluten into two or more fractions which differed progressively in solubility, molecular shape or symmetry, and in amide and arginine nitrogen. The evidence available has been interpreted by some of the above investigators as indicating that gluten is made up of relatively few distinct protein components and by McCalla and associates as showing that "the main gluten protein is a single complex that can be divided into many fractions differing systematically in both physical and

[1] Cooperative investigations between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, and Department of Agronomy, Ohio Agricultural Experiment Station.

[2] Chemist, Division of Cereal Crops and Diseases at the Federal Soft Wheat Laboratory, Wooster, Ohio.

chemical properties." Haugaard and Johnson (1930) believe that the protein complex may change reversibly depending on conditions of temperature, salt content, hydrogen ion concentration, etc. Blish (1936), however, has found contradictory evidence supporting the existence of a single protein complex and a mixture of a few distinct individual proteins.

Much of the earlier work with protein separates dealt with the aqueous-alcohol-soluble fraction, gliadin. Gliadins differing in solubility, viscosity, and chemical composition were obtained by Cook (1931) using different methods of preparation and by Haugaard and Johnson (1930) using fractionation. The latter also found differences in optical activity. Sinclair and Gortner (1933) could not confirm Haugaard's and Johnson's differences in chemical composition, however. Differences in molecular weight of gliadin fractions were obserbed by Krejci and Svedberg (1935).

Viscosity measurements have been used to a limited extent in the characterization of gluten fractions, especially gliadin, and these correlated with solubility. The fact that there has been reported no systematic application of viscosity to all fractions of gluten is doubtless at least partly due to the difficulty in interpreting the data until recent years. More recently, however, Lauffer (1942) has summarized the information available and showed that the rate of change of viscosity with concentration of solute is related to the ratio of molecular length to thickness (axial ratio or symmetry) and that molecular hydration is a minor factor. Since rate of change in viscosity to concentration is related to the absolute viscosity at a given concentration, it follows that solutions of similar material at equal concentrations but different viscosity indicate a difference in axial ratio of the solute molecules. Thus viscosity data can be used to characterize gluten fractions by indicating average molecular or aggregate shape.

Materials and Methods

The flours used were obtained from composites of pure wheat varieties ground in an experimental mill. The wheat had been grown at various agricultural experiment stations in Kansas, Nebraska, and Colorado. Unless otherwise stated, the dispersions were obtained from gluten which was washed in the 6.8 pH, 0.1% phosphate buffer solution of Dill and Alsberg (1924). Viscosity measurements were made with Ostwald pipettes at 30°C, standardized with water, 20%, and 40% sucrose. The two dispersion media were 50% alcohol and 50% acetic acid. These were made up by volume rather than by weight.

Critical peptization temperature (C.P.T.) was described by Dill and Alsberg (1924) and as used in the present study is the temperature

at which a 2% protein solution in 50% alcohol showed marked opaqueness on slow cooling.

The method of purifying gliadin outlined by Dill and Alsberg (1924) consists essentially of two procedures which will be referred to as "cooling" and "foam" precipitations. The cooling precipitation as used consists of storing an alcoholic 2% protein solution at 4°C for 48 hours. During this time a viscous, honeylike layer formed at the bottom of the flask leaving a cloudy solution which became sparkling clear at room temperature. A double cooling precipitation is one cooling precipitation followed by another applied to a 2% protein solution of the first "honey" layer. The foam precipitation consisted of diluting slowly and shaking vigorously a 2% protein aqueous alcohol solution with five volumes of water containing 0.1% NaCl. Part of the protein was precipitated in the foam and part dissolved in the cloudy solution. The protein in the foam was redissolved readily by adding 95% alcohol. Evaporation of the protein solution was necessary, and was found not to affect resolution or viscosity if the temperature was kept below 40°C. Solutions containing salt were dialyzed and redissolved before viscosity measurements were made. Viscosity values were determined at 2% protein in 50% alcohol unless otherwise indicated. Viscosity measurements for calculations of axial ratio were made on dispersions containing 0.05 to 0.075 *M* NaCl to minimize the electroviscous effect as pointed out by Cohn and Edsall (1943).

Results

Aqueous alcohol extracts of gluten from different samples of hard winter wheat were found to have similar viscosities, but samples of gliadin purified by slightly different methods were widely different in viscosity even for the same samples. Conversely, preparations by the alternate cooling and foam precipitation method of Dill and Alsberg (1924) produced gliadin of the same viscosity from flour samples known to differ widely in protein quality.

A qualitative study of the fractions resulting from cooling and foam precipitations, as diagrammed in Figure 1, led to information which explained the above results. Two successive extractions of gluten were combined and subjected to a double cooling precipitation. The combined clear solutions contained 26% of the protein, which had a viscosity of 2.74 centipoises. The "honey" layer contained the remainder, or 74%, and had a viscosity of 3.03 centipoises. A foam precipitation separated the honey layer into a soluble portion with a viscosity of 2.87 centipoises containing 24% of the original protein and the foam sediment with a viscosity of 3.09 centipoises containing 43% of the original protein. After finding that a cooling precipitation would not

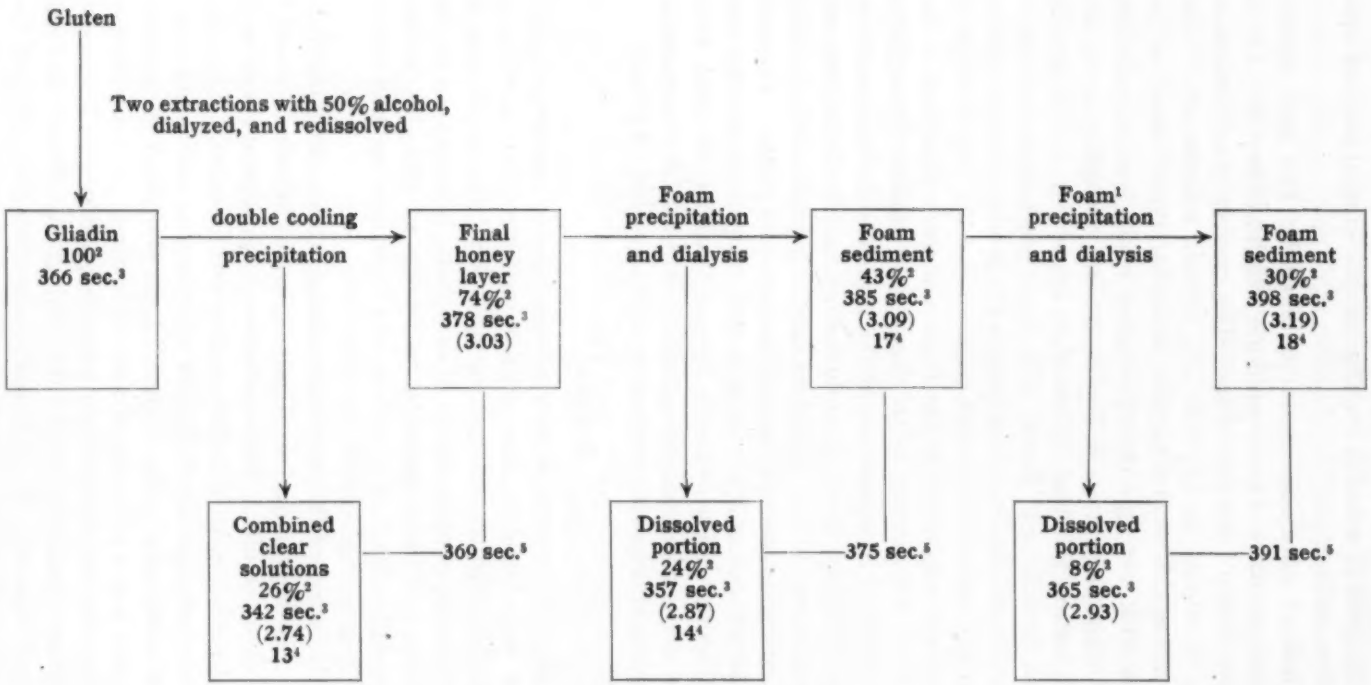

Fig. 1. Schematic plan used in fractionating gliadin.

[1] After finding that a cooling precipitation was not capable of further fractionation.
[2] Values expressed are the amounts of original gliadin in the particular fraction.
[3] Viscosities of 2% protein solutions measured in seconds. Those values in parentheses are the same values given in centipoises.
[4] Calculated axial ratio.
[5] Calculated viscosity of a mixture of the two fractions in original proportions.

fractionate the foam sediment further, it was subjected to another foam precipitation. The resulting soluble portion and foam sediment contained 8% and 30% of the original protein and had viscosities of 2.93 and 3.19 centipoises, respectively.

Since previous work had shown that the viscosity of a mixture of two gliadin fractions was approximately proportional to the relative amounts and viscosity of each fraction, the viscosities of the mixtures of the above fractions in their original proportions were calculated and are given in Figure 1. The agreement of these values with those of the original fractions shows that there was no change in the first four fractions and very little if any in the last two.

The results demonstrate that both the cooling and foam precipitations separate gliadin into fractions differing in solubility and in viscosity. It is easy to understand how gliadins of different viscosity could be prepared from the same original alcoholic extract of gluten by varying the order or number of foam and cooling precipitations, the fractions discarded, the conditions of concentration, temperature, or salt content. Likewise by rigid adherence to procedure and conditions it is possible to prepare gliadin of identical viscosity from gluten extracts that differ considerably.

In the Dill and Alsberg method of purifying gliadin, the high viscosity material is discarded in the cooling precipitation but saved in the foam precipitation. Therefore the final material is a gliadin of medium viscosity which has been separated from other gliadins of both higher and lower viscosity.

The application of Simha's equation (1940) for rodlike ellipsoids to rate of change of viscosity with concentration as outlined by Lauffer (1942) indicates that the molecules of the above fractions with viscosities of 2.74, 2.87, 3.09, and 3.19 centipoises, compared to 2.04 centipoises for 50% alcohol, are, respectively, 13, 14, 17, and 18 times as long as thick. In comparison, Neurath (1939) and Mehl, Oncley, and Simha (1940) obtained axial ratios ranging from 10.5 to 11.1 for purified gliadin calculated from viscosity, sedimentation, and diffusion data. The solubility of these fractions thus appears to be related to the axial ratio or molecular symmetry, the more symmetrical molecules being the more soluble in dilute salt solutions and cold 50% alcohol.

Further evidence of the heterogeneity of gliadins purified by various methods was obtained by determining the critical peptization temperature (C.P.T.) of a number of fractions. In the fractions known to be *fairly* homogeneous as the result of a series of cooling and foam precipitations, the C.P.T. was closely related to viscosity, that is high C.P.T. with high viscosity. This relationship did not hold for relatively unfractionated gliadin solutions. This lack of agreement appeared to be

due to the fact that in this case the C.P.T. indicates the insolubility of only a small portion of the total protein present. Since viscosity expresses the average of all the proteins present, C.P.T. and viscosity would be correlated in the more homogeneous mixtures only.

Since the results obtained by cooling and foam precipitations and the critical peptization temperature study indicate that the aqueous alcohol-soluble protein, gliadin, is heterogeneous, it might be expected that extraction of flour at different temperatures would produce gliadin of varying solubility and viscosity. Accordingly an experiment was set up in which 50-g subsamples of flour were extracted four times with 200 cc of 50% alcohol at − 12°, 4°, 27°, and 60°C. Centrifuging or filtering in between extractions was also carried out at these temperatures. At the three lower temperatures agitation was continuous and the extracts were centrifuged. At 60°C agitation was intermittent and the extracts were filtered. The amount of protein obtained by each extraction decreased progressively and in the fourth the flour seemed to be nearly exhausted of soluble protein. Since some of the material extracted at 60°C was not soluble in aqueous alcohol at 30°C, another solvent was required. Since both gliadin and the simpler glutenin proteins could be dispersed in 50% acetic acid, the extracted proteins were dispersed in this solvent for comparison of viscosities. Although proteins hydrolyze slowly in this dispersion medium (Cook and Rose, 1935), the amount of change in viscosity is slight in the time required to complete the analysis and viscosity determinations. It was found that the viscosity of alcohol-soluble flour proteins in 50% acetic acid was a linear function of that in 50% alcohol with a correlation coefficient of + 0.95 for 30 pairs.

The results of the extraction are plotted in Figure 2 in which the percent of the total protein obtained by the four extractions and the viscosity of the 2% protein solutions in 50% acetic acid, after dialysis and evaporation, may be compared to the temperature of extraction. Solubility at 60°C or higher temperatures is doubtless limited, owing to denaturation which probably takes place in the more complex molecules. Electroviscous effects were not minimized in these dispersions, and therefore axial ratios have not been calculated from these data.

The data show how the amount as well as the viscosity of the protein dissolved or dispersed is dependent on the temperature. Since viscosity is indicative of the symmetry of the molecules, the less symmetrical the molecule the less is its dispersibility. By common usage gliadin and glutenin are considered to be the major protein constituents—i.e. the aqueous alcohol, room temperature soluble and insoluble fractions, respectively. However, by increasing the temperature to 60°C, about one fourth of the glutenin is dispersible. The increase in

viscosity of the 60°C extract compared with that obtained at 27°C indicates that this soluble glutenin is less symmetrical than the gliadin fraction.

Whole gluten will disperse slowly in 50% acetic acid, forming a smooth dispersion, provided it is agitated and centrifuged. About 10 to 15% of the protein is generally not dispersed sufficiently to be included in the centrifugate. Viscosity measurements can be made on these dispersions although they are not satisfactorily reproducible from one dispersion to another and they show some plastic flow. Hydrolysis as measured by viscosity changes is not detectable if the

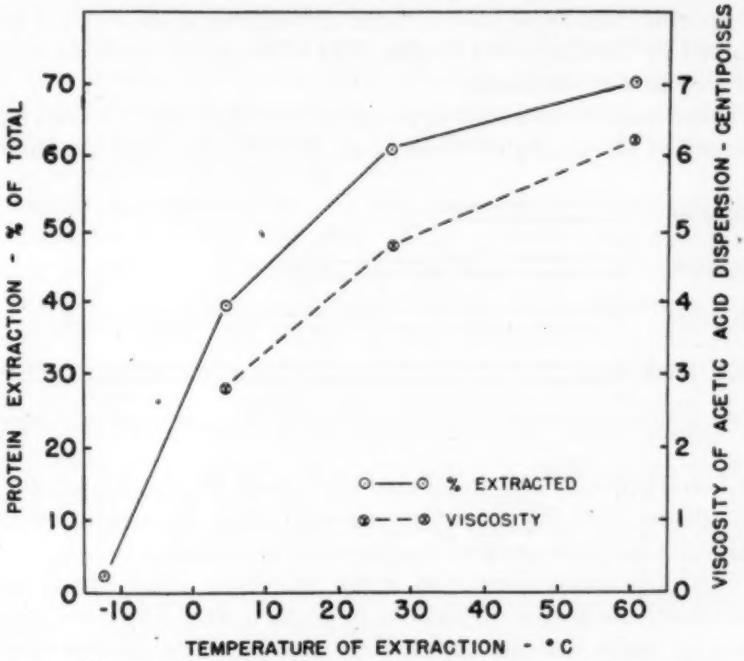

Fig. 2. Amount and viscosity of protein extracted from flour at various temperatures.

dispersion is stored at − 12°C during the dispersion period. In general the viscosity of such gluten dispersion is about 10.5 centipoises, compared to 1.62 centipoises for 50% acetic acid, which corresponds to an average axial ratio of 41. Since the viscosity of a mixture of two protein dispersions is approximately proportional to their viscosities and the amounts mixed, the viscosity of glutenin or the nonalcohol-soluble protein of flour may be approximated. On the basis of 50% of the flour protein being gliadin of axial ratio of 19, the glutenin would have an axial ratio of 47. This adds additional evidence to that of the extraction experiment that the protein insoluble in the 50% alcohol is more complex than the soluble portion.

Discussion

In calculating axial ratios it was necessary to assume that the shape of the gluten protein molecules was rodlike ellipsoids of revolution, as is pointed out by Lauffer (1942) for gliadin, rather than platelike ellipsoids. pH has been assumed to be of no consequence since the addition of electrolytes reduces the charge on the micellae to such an extent that it does not introduce an important error in viscosity (Lauffer, 1946). It is assumed that hydration plays an unimportant role, compared to molecular shape, in the viscosity of these dispersions (Lauffer, 1942), and it has therefore been left out of the calculations. The specific volume of 0.73 cc used throughout is an average of that obtained by McCalla and Gralén (1942) for gluten proteins in acetic acid and alcohol solutions.

These assumptions and approximations may affect somewhat the accuracy of the values obtained; but since the fractions examined are

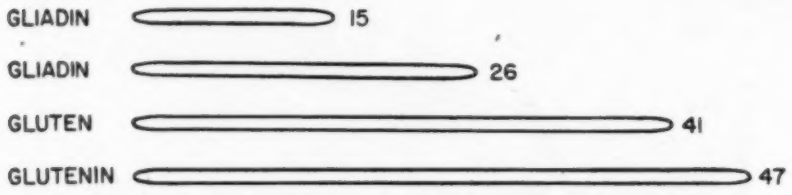

Fig. 3. Proportionate length vs. thickness of various gluten fractions according to results.

still heterogeneous and indefinite and could be duplicated only by rigid adherence to the procedure of preparation, the absolute values of axial ratios are unimportant compared to the relative values.

The dispersions contained some materials other than protein, particularly the gluten dispersions in acetic acid. The main impurity was very finely divided starch that could not be thrown down by centrifuging. However, since the viscosity of these dispersions was high, the amount of starch present did not contribute an appreciable error relative to viscosity. The solutions of the aqueous alcohol-soluble proteins were considerably purer, on the basis of their nitrogen content, and did not contain suspended starch and only a few hundredths of a percent of nonprotein material.

As illustrated in Figure 3 the shape of the wheat protein molecule appears to vary from relatively short ellipsoids, which are about 15 times as long as thick, to the relatively long ellipsoids, which appear to be about 47 times as long as thick (based on the data from the acetic acid dispersions). As the molecular length relative to thickness increases from approximately 15 to 26, the temperature of 50% aqueous

alcohol has to be increased above 4°C in order to get solubility. Increases in temperature above 27°C will dissolve protein of slightly longer relative molecular length. Still longer molecules will disperse in 50% acetic acid, as do the short ones, but there appears to be a small percentage of molecules too complex to disperse even in acetic acid unless complexity is reduced by allowing time for hydrolysis.

Viscosity data alone do not indicate whether the increase in axial ratio is due to a difference in molecular weight or to a lengthening of the molecules of equal molecular weight. Changes in axial ratio could also be due to aggregation of simpler molecules. However, no gliadin seems to be formed in the slow hydrolysis of glutenin by acetic acid, which is some evidence that these are not gliadin aggregates. McCalla and Gralén (1942) have shown that the molecular weight of the gluten protein varies with solubility, and since solubility and viscosity are highly related the increase in axial ratio may be due, at least in part, to an increase in molecular weight.

It seems reasonable to assume that the solubility in sodium salicylate found by McCalla and Rose (1935) parallels the solubility in aqueous alcohol and acetic acid. If this is the case, as the molecule increases in length it decreases in solubility in three solvents, increases in molecular weight, and except for the most soluble 10–15% it increases in arginine nitrogen and decreases in amide nitrogen.

All fractionation, viscosity, and solubility data obtained in the work reported here support the contention that wheat gluten "is a single protein complex that can be divided into many fractions differing systematically in both physical and chemical properties." However, the possibility of change in molecule size or shape postulated by Sinclair and Gortner (1933), Haugaard and Johnson (1930), and Blish (1936) by treatment with electrolytes, hydrogen ions, precipitation technic, etc., has not been eliminated.

Summary

Wheat flour gluten has been fractionated into components differing progressively in viscosity and solubility. The differences in viscosity have been interpreted to indicate differences in axial ratio of rodlike ellipsoidal molecules.

The molecules of the protein commonly called gliadin appear to be the most symmetrical and the most soluble. However, some of these molecules appear to be twice as unsymmetrical as others. Glutenin molecules likewise vary in symmetry and are more unsymmetrical than those of gliadin. The more symmetrical glutenin molecules are soluble in hot aqueous alcohol, but insoluble at room temperature. Symmetry and solubility in several solvents appear to be related, the

more symmetrical the molecule the greater the solubility or dispersibility. The data add further evidence to that in the literature that gliadin and glutenin are part of a complex protein system differing systematically in physical and chemical properties with no clear distinction between the two.

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KINETICS OF BETA-AMYLASE ACTION IN 20% STARCH PASTES AT ELEVATED TEMPERATURES

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The kinetics of amylase action are discussed in various degrees of complexity in the literature (Kneen, 1944), and a good description of elementary principles of hydrolytic enzyme action is given by Van Slyke (1942). The kinetics of diastase action in particular go back to the work of Kjeldahl (1879) and of Lintner (1886), and to a more comprehensive study by Brown and Glendinning (1902) of the saccharifying action of diastase on starch. Wohlgemuth (1908) described the dextrinizing action of diastase on starch. More recent studies have made use of these basic principles to measure the strengths of alpha- and beta-amylases individually (Claus, 1946). In general, however, amylase kinetics have been studied in 2–3% starch pastes, and at temperatures well below those which cause inactivation of the enzymes. But the brewer is interested in what occurs when malt amylases act on starch concentrations approximating those in a brewing mash (about 20%) and at brewing temperatures of 50°–80°C, in the range which causes rapid inactivation of the amylases. This report covers the first phase of investigations on the kinetics of purified malt amylases acting on 20% corn starch pastes at elevated temperatures.

Apparatus and Procedure

The experiments were carried out on 20% corn starch pastes which had been liquefied with pure alpha-amylase. The enzyme was added to the starch milk which was rotated in a 500-ml mixing flask (an Erlenmeyer with vertical vanes blown into it) in a water bath, and brought up through a definite time-temperature schedule to gelatinize and liquefy the starch. The solution was then boiled 20 minutes, buffered with acetate, and brought to weight with water. The finished substrate was 20.8% starch by weight.

To convert, 48 g of substrate was weighed into a 125-ml mixing flask, which was then rotated by a hollow shaft in a water bath, as shown in Figure 1. The beta-amylase, in 2 ml of solution, was introduced below the surface of the starch with a pipette, and rotation of the flask produced rapid mixing. The resulting mixture was 20% starch.

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From time to time during the conversion, 1.5–2.0 g samples of the mixture were withdrawn directly into weighed 50-ml flasks containing 10 ml of 0.2% sodium hydroxide solution which instantly stopped the enzyme action. The flasks were then reweighed to ascertain the amount of the sample to the nearest milligram. Each withdrawal was made with a clean, dry tube system as shown in Figure 1. Further dilution was then made with the alkali solution, on an analytical balance, so that the diluted solution contained 0.5 g of converted substrate per 5.0 ml of solution. 5.0 ml aliquots were titrated with 10 ml of ferricyanide solution (Hildebrand and McClellan, 1938) and 0.05 *N*

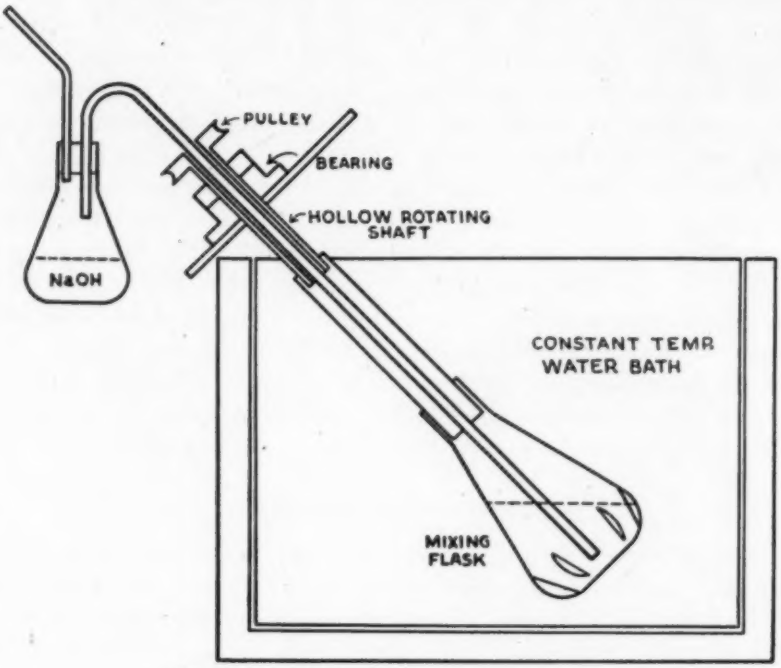

Fig. 1. Schematic diagram of conversion apparatus.

ceric sulfate solution to determine the reducing power of the aliquot. Careful calibration showed that 1.0 ml of 0.05 *N* ceric sulfate solution corresponded to 1.59 mg pure maltose (corrected for hydrate) in the presence of 0.5 g of the substrate.

Titrations were carried out serially, one tube being prepared every 4 minutes, heated in a boiling water bath 20 minutes, cooled 4 minutes, then titrated. These titrations were accurate to within a few hundredths of a milliliter of ceric sulfate solution, so that duplicate titrations were rarely carried out. Much more dependable results were obtained by preparing more samples and titrating singly.

Theoretical Considerations

The Reciprocity Relationship. If the reciprocity relationship is valid, the time required for a given amount of enzyme to produce a definite amount of action (such as an arbitrary amount of reducing power) is inversely proportional to the amount of enzyme. To test the validity, conversions were carried out with various small amounts of beta-amylase, and samples were withdrawn very soon after the addition of the enzyme.

Several such experiments were performed, with results consistently like those shown in Figure 2. In the experiment shown, 1.65 and 3.30 K-S units of beta-amylase (Kneen and Sandstedt, 1941) were added

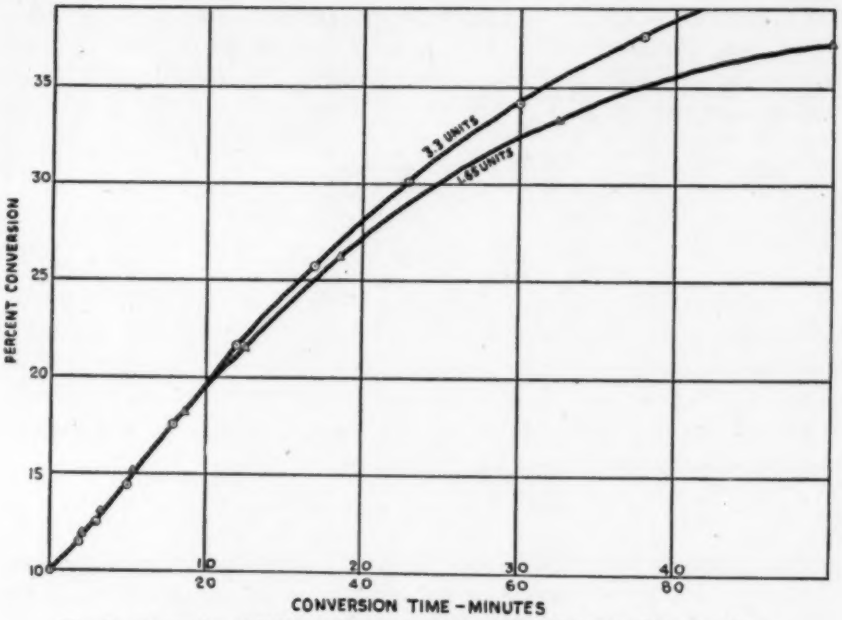

Fig. 2. Conversion of 20% starch paste by 3.30 and 1.65 K-S units of beta-amylase, at 40°C and pH 5.5, showing failure of reciprocal relationship.

to two simultaneous conversions at 40°C. In the figure, the lower time scale is for the smaller amount of enzyme (lower curve), and the upper time scale is for the larger amount of enzyme (upper curve). If the reciprocity relationship were valid for all degrees of conversion, the two curves would be superimposed. Actually, they coincide only up to about 17–18% conversion, including 10% due to liquefaction of the substrate. Within this small range, conversion is proportional to time and to amount of enzyme. That is, it corresponds to a zero-order reaction, and appears to offer the best field for elementary studies of beta-amylase kinetics.

Determination of "Activity" and "Inactivation Index" from Experimental Data. Assuming that beta conversion does proceed according to a zero-order reaction, one may write that the increment in the amount of maltose, dM, in time dt, is proportional to the amount of active enzyme, β, and to its activity, A. That is:

$$dM = A\beta dt \tag{1}$$

Suppose that the amount of active enzyme, β, at any time, t, is the result of logarithmic inactivation by the heat, or that

$$\beta = Be^{-kt} \tag{2}$$

where B is the amount of enzyme added at the beginning of the experiment (when $t = 0$) and k is the temperature inactivation index, which may also be thought of as the logarithm of the fraction of active enzyme which is inactivated per unit of time. Substituting (2) in (1) and integrating, we have

$$\int_{M_0}^{M} dM = \int_0^t ABe^{-kt}dt \tag{3}$$

$$M - M_0 = AB(1 - e^{-kt})/k \tag{4}$$

M_0 represents the amount of maltose present when $t = 0$ (reducing power of the substrate calculated as maltose) and M is the amount of maltose after conversion for time t. The difference, $M - M_0$, which is the significant value, may be represented as ΔM. If the conversion is carried out at a sufficiently low temperature so that no inactivation occurs, k is zero, and equation (4) becomes:

$$\Delta M = ABt \tag{5}$$

which is the equation of a straight line. Experimentally, it is observed that the conversion curves are straight lines for temperatures below 45°C, in which case the value of activity A is easily calculated from the slope of the observed curve (Figure 3).

If M is calculated from experimental data as grams of maltose produced during the conversion, and B is expressed in terms of K-S units, then the enzyme activity A will be expressed as grams of maltose per K-S unit per unit of time—minute or hour.

The calculation of A and k from experimental data and equation (4) is relatively simple. The value of k depends on the *shape* of the curve, and A upon the *amount* of reducing power. In practice, it is simpler to make calculations from experimental data in terms of milliliters of ceric sulfate solution required for titration, milligrams of enzyme used, and minutes of conversion time. Values of A so calculated can be

changed into whatever units are desired by multiplying by a suitable factor. Calculations are made as follows. Equation (4) takes the form:

$$RP_1 = AB(1 - e^{-kt_1})/k \tag{5a}$$

$$RP_2 = AB(1 - e^{-kt_2})/k \tag{5b}$$

where RP represents the increase in reducing power in time t, in terms of ml 0.05 N ceric sulfate solution. It is most convenient to choose values of conversion time such that $t_2 = 2t_1$. Then

$$e^{-kt_2} = e^{-2kt_1} = (e^{-kt_1})^2$$

Using this expression and dividing equation (5b) by (5a), one obtains

$$e^{-kt_1} = RP_2/RP_1 - 1 \tag{6}$$

From experimental curves of RP plotted against time, and use of equation (6), a number of values of k can be calculated along the course of the curve. The average value of k is taken as the "temperature inactivation index." From it the "half-life" of the enzyme can be calculated by the formula:

$$\text{Half-life} = 0.692/k \tag{7}$$

In several dozen experiments, the values of k as calculated from various parts of an observed curve were remarkably constant, indicating beyond doubt that the assumption of logarithmic destruction of the enzyme by heat is valid.

With the value of k established for any given conversion curve, one substitutes it in equation (5a) and calculates a number of values of A from various values of RP on the curve. The average A is taken as the value for that experiment. Now with known values of A and k, one can calculate the entire RP–t curve, and determine how well it fits the experimental points.

An illustrative set of calculations is given in Table I and shown graphically in Figure 3, in which the solid line represents the calculated curve, and the points are experimental. Especially to be noted in the table is the constancy of the various values of A and k, and the excellent agreement between calculated and observed values of reducing power. This one is by no means an exceptional experiment.

Experimental Results

Activity and Temperature Inactivation as a Function of pH, at a Conversion Temperature of 65°C. Values of activity A and of the temperature inactivation index k were determined for a number of values of pH between 4.68 and 6.85, all at a conversion temperature

TABLE I

CONVERSION AT 60°C, pH 5.5, WITH 5 MG[1] BETA-AMYLASE

Conv. time (mins.)		*RP* (ml 0.05 *N* ceric sulfate)			*k*	*A*[5]
t_1	t_2	Obs.[2]	Smooth[3]	Calc.[4]		
2	4	0.63	0.63	0.630	—	0.0667
3	6	—	0.92	—	0.0592	—
4	8	1.18	1.18	1.191	0.0568	0.0661
5	10	—	1.45	—	0.0607	—
6	12	1.73	1.69	1.689	0.0584	0.0668
7	14	—	1.92	—	0.0589	—
8	16	2.12	2.12	2.132	0.0564	0.0664
9	18	—	2.33	—	0.0589	—
10	20	2.52	2.52	2.525	0.0602	0.0666
12	—	2.88	2.88	2.875	—	0.0668
14	—	3.24	3.21	3.187	—	0.0672
16	—	3.40	3.47	3.464	—	0.0669
18	—	3.70	3.74	3.710	—	0.0673
20	—	3.95	3.92	3.929	—	0.0666
				Average	0.0587	0.0667[5]

[1] 1.0 mg beta-amylase equivalent to 0.165 K-S units.
[2] Experimentally observed *RP* at conversion time t_1.
[3] *RP* from best smooth curve through experimental points, used to calculate *k* and *A*.
[4] *RP* calculated for t_1 using average values of *k* and *A*.
[5] *A* in terms of grams maltose per K-S unit per hour = 0.0667 × 57.84 = 3.858.

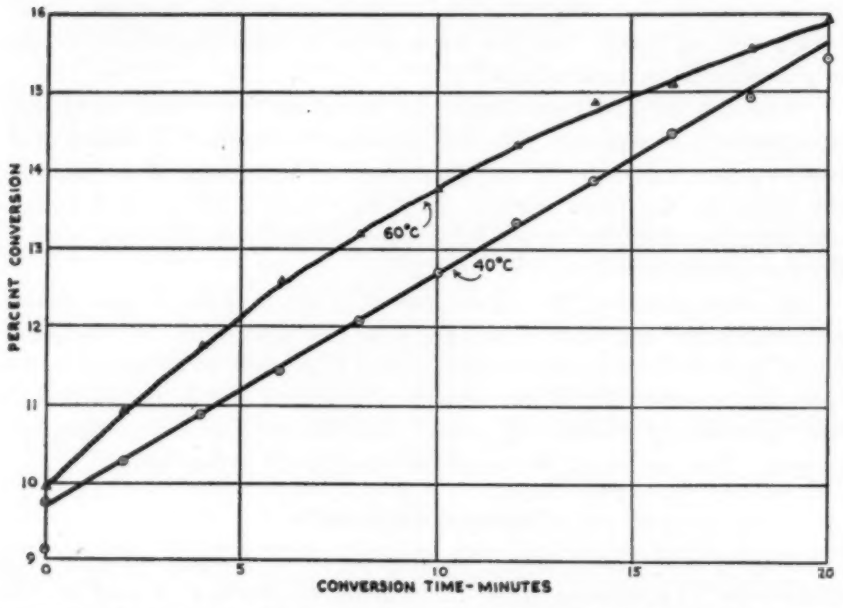

Fig. 3. Conversion of 20% starch paste by beta-amylase at pH 5.5. Lower curve at 40°C; inactivation $k = 0.0$, activity $A = 1.88$. Upper curve at 60°C; inactivation $k = 0.0587$, activity $A = 3.858$ (Table I).

of 65°C which is approximately the optimal temperature for our beta-amylase preparations.

The results are summarized in Table II and are graphed in Figure 4. Values of activity A are given in terms of grams of maltose per K-S unit per hour to show how beta activity under these conditions of substrate concentration, temperature, and pH compare with beta

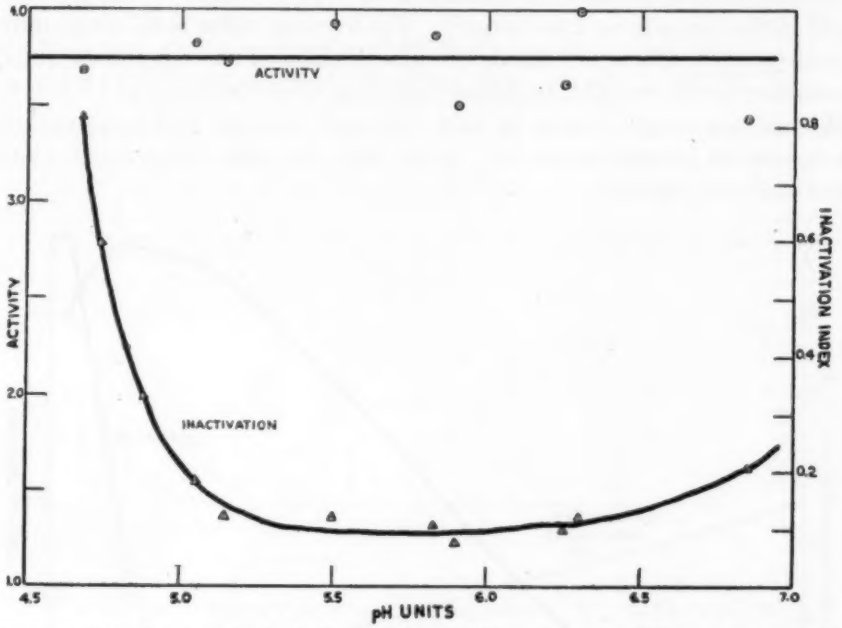

Fig. 4. Activity A and inactivation k of beta-amylase in 20% starch paste at 65°C, as a function of pH. Left-hand ordinate scale for activity curve, right-hand ordinate scale for inactivation curve.

TABLE II

ACTIVITY AND INACTIVATION OF BETA-AMYLASE AT 65° C AS A FUNCTION OF pH

pH	Activity[1] A	Inactivation[2] k	Half-life (mins.)
4.68	3.69	0.810	0.85
4.80	3.52	0.278	2.49
4.90	3.49	0.235	2.94
5.05	3.82	0.180	3.86
5.15	3.73	0.121	5.70
5.50	3.93	0.125	5.55
5.83	3.87	0.106	6.56
5.90	3.50	0.078	8.91
6.25	3.62	0.100	6.92
6.30	4.10	0.123	5.63
6.85	3.44	0.207	3.35

[1] Grams maltose per hour per K-S unit of beta-amylase.
[2] Per minute.

activity under the conditions which define unit activity (K-S unit)—that is, 2% starch solution, 30°C, pH 4.63, resulting in the production of maltose at the rate of one gram per hour per unit of beta-amylase. Values of k are given in terms of the logarithm of the fraction of enzyme inactivated per minute.

Although the graph shows a certain amount of scatter for the activity values, there is no evidence that activity tends to change with pH in the range from 4.68 to 6.85. The average value is 3.7 times unit activity. On the other hand, the inactivation of the enzyme is quite sensitive to pH outside the broad minimum between about pH 5.4–6.4. Within this range, it may be said that both activity and inactivation rate remain sensibly constant. This range for other temperatures has not been determined.

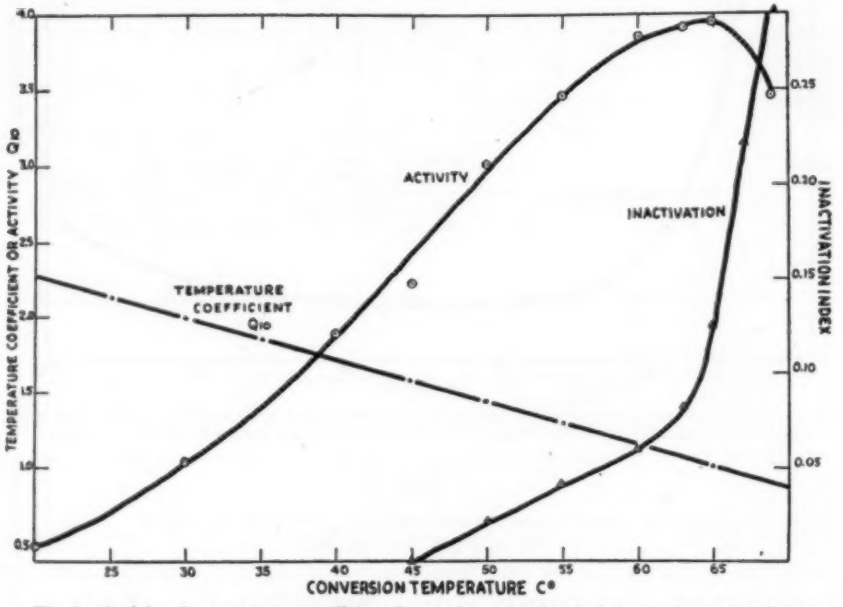

Fig. 5. Activity A, temperature coefficient Q_{10}, and inactivation k of beta-amylase in 20% starch paste of pH 5.5, as a function of conversion temperature. Left-hand ordinate scale for activity and temperature coefficient; right-hand ordinate scale for inactivation.

Activity and Temperature Inactivation as a Function of Temperature, at pH 5.5. Values of A and k were determined at 11 different conversion temperatures between 20°C and 69°C, all in substrates buffered at pH 5.5. The results are summarized in Table III, and graphed in Figure 5. Values of activity (an intrinsic function of the *live* enzyme, not to be confused with over-all converting power which includes the effects of inactivation) are found to rise to a maximum at about 65°C, and to decrease at higher temperatures. Of interest, though perhaps

fortuitous, is the fact that the activity of one K-S unit of beta-amylase is still 1.0 at 30°C, even though substrate concentration and pH are quite different from standard.

Values of the temperature coefficient of activity, Q_{10}, are also shown. These values were determined from the activity-temperature curve by drawing a tangent to the curve at the temperature shown, and calculating the ratio of activities indicated by the tangent at temperatures

TABLE III

ACTIVITY AND INACTIVATION OF BETA-AMYLASE AT PH 5.5 AS A FUNCTION OF TEMPERATURE

Temp.	Activity[1] A	Temp. coefficient Q_{10}	Inactivation[2] k	Half-life (mins.)
20°C	0.49	—	0	Infinite
25	—	2.14	—	—
30	1.04	2.00	0	Infinite
35	—	1.85	—	—
40	1.88	—	0	Infinite
45	2.22	1.59	0	Infinite
50	2.99	1.46	0.021	32.4
55	3.46	1.29	0.041	17.1
60	3.86	1.15	0.059	11.8
63	3.91	—	0.081	8.6
65	3.93	1.00	0.125	5.6
67	(4.25)[3]	<1	0.221	3.1
69	3.46	<1	0.292	2.4

[1] Grams maltose per hour per K-S unit of beta-amylase.
[2] Per minute.
[3] Obviously high—omitted from Figure 5.

5°C above and below the point of tangency. Q_{10} as thus calculated is really a *rate* of change of activity per 10°C, rather than a simple ratio of activities at temperatures 10°C apart. These values of Q_{10} fall remarkably well on a straight line,[1] from a value of 2.14 at 25°C to a value of 1.0 at 65°C, and dropping below 1.0 at higher temperatures.

Inactivation of the enzyme by heat does not take place at pH 5.5 at temperatures below about 45°C. Inactivation occurs above 45°C,

[1] This fact permits determination of the equation of the curve for activity vs. temperature. Values of Q_{10} as calculated above are given by the expression

$$\left(A + \Delta T \frac{dA}{dT}\right) \Big/ \left(A - \Delta T \frac{dA}{dT}\right)$$

where ΔT = 5°C above and below the point of tangency. The equation of the straight line for Q_{10} vs. T is

$$Q_{10} = K - cT$$

where K has the experimental value 2.8525 and c is 0.0285. Upon integration of the equation

$$\frac{A + \Delta T \frac{dA}{dT}}{A - \Delta T \frac{dA}{dT}} = K - cT$$

one obtains

$$A^{c\Delta T} = C(K + 1 - cT)^{\frac{1}{c}} e^{-(K+1-cT)}$$

C is the constant of integration having the empirical value 2.25. This expression fits the activity-temperature curve of Figure 5 with a high degree of precision, and indicates that it may be theoretically possible to determine activities at all temperatures from a few experimental points at convenient temperatures.

and the curve of k against temperature shows a sharp increase in the rate of inactivation at 63°–65°C, corresponding approximately with the temperature of maximum activity. 63°–65°C may therefore be considered as a very critical point in the enzyme-starch reaction at pH 5.5.

Optimal Conversion Temperatures. The optimal temperature for a conversion cannot in general be determined except in conjunction with a definite conversion time. It is interesting to calculate, with the help of equation (4) and the values of A and k from Table III, what

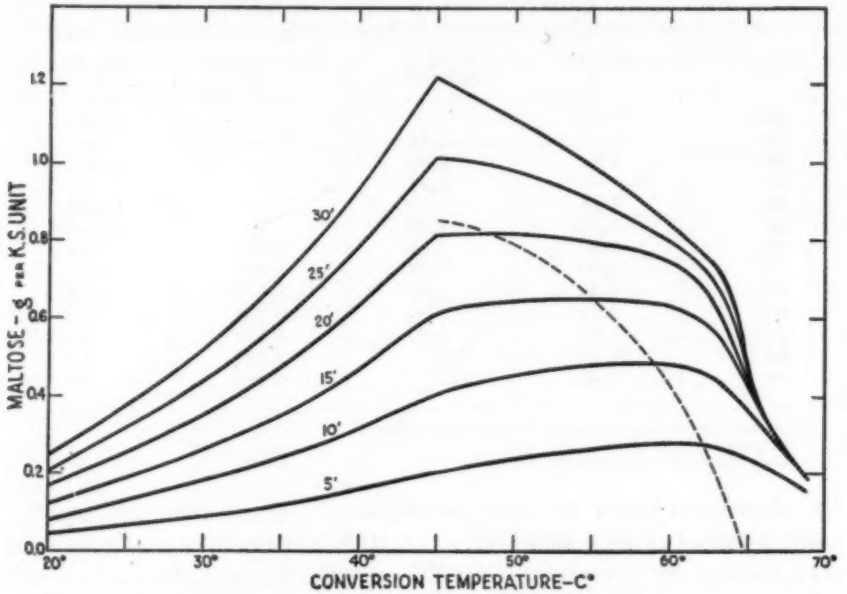

Fig. 6. Saccharification of 20% starch paste of pH 5.5 by beta-amylase, as a function of time and conversion temperature. The dotted line indicates the shift of maximum saccharification toward higher conversion temperatures as conversion time is decreased.

would be the amount of maltose produced per unit of beta-amylase under these conditions, for various conversion times and temperatures. The results are shown graphed in Figure 6. It is seen that for all periods above about 20 minutes, 45°C is the optimal temperature, but for shorter periods during which inactivation occurs and saccharification does not proceed so far, the optimal temperature shifts to higher values to make use of the larger values of A.

Summary

Conversion of 20% starch pastes by beta-amylase has been studied at pH 5.5 and at temperatures between 20°C and 69°C; also at 65°C and at pH values between 4.68 and 6.85.

Under these conditions, production of maltose takes place according to a zero-order reaction up to about 18% conversion. The enzyme is inactivated exponentially by temperatures above 45°C. Mathematical treatment of conversion curves permits determination of the intrinsic activities of the enzyme, and of its temperature inactivation indices.

Activity at 65°C remains constant over the pH range studied, while inactivation is at a minimum between pH 5.4 and 6.4, increasing on either side.

Activity at pH 5.5 increases eight-fold between 20°C and 65°C, at which temperature it reaches a maximum and begins to decrease.

The temperature coefficient Q_{10} of activity decreases linearly from 2.14 at 25°C to 1.0 at 65°C and to less than 1.0 at higher temperatures.

Temperature inactivation sets in at 45°C and becomes greater as temperature increases. The curve shows a sharp rise at about 63°–65°C, corresponding approximately with the activity maximum.

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SOURCES OF BETA-AMYLASE AS SUPPLEMENTS TO BARLEY MALTS IN SACCHARIFICATION AND FERMENTATION[1]

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Increase in the use of industrial and beverage alcohols has resulted in the much-increased use of diastatic material other than malt, such as "mold bran." There are some processes where such fungal and bacterial material may not be desirable. Possible nonmicrobial sources which could be used as supplements to malt are sulfite solution extracts of flour (Balls and Tucker, 1943) and sweet potatoes (Balls, Thompson, and Walden, 1946). In contrast to the microbial amylases, these sources are practically devoid of alpha-dextrinogenic activity, the total amylolysis being due to the beta-amylase type of enzyme.

In a study of the alpha- and beta-amylase of malts from many varieties of barley, Nelson and Dickson (1942) found that, in general, high diastatic power is associated with high values in both alpha and beta components, whereas malts of California barley varieties showed very low beta values, but normal to high alpha values. Obviously, this discrepancy may be remedied by the addition of sources rich in beta-amylase such as wheat and sweet potatoes.

Beta-amylase is generally regarded as the component in malt principally responsible for saccharification, but it is also evident that its action is not only facilitated but greatly extended by the presence of alpha-amylase. Thus Kneen, Beckord, and Sandstedt (1941) estimated after comparing the starch-degrading properties of 12 barley malts that over three-quarters of the degree of saccharification obtained was due to beta-amylase. But they also pointed out that several malts high in alpha-amylase showed greater saccharogenic activity than would be predicted on the basis of the beta-amylase content. There was, however, no correlation between alpha-amylase and saccharogenic activity.

The necessity of alpha-amylase for the practical saccharification of starch is obvious from the fact that this enzyme is the characteristic amylase of malted grains. Kneen (1944) found that the ungerminated grains of a number of cereals possess alpha-amylase activity in measurable quantities; but the amount reported is very small compared to that developed during germination. Recently Balls and Schwimmer

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(1944) and Schwimmer (1945) have shown that raw starch is completely digested by alpha-amylase of animal origin free from beta-amylase, provided the end products of the reaction are removed. This removal could be accomplished by dialysis or by the action of added maltase. Beta-amylase, on the other hand, has little or no effect on raw starch under similar conditions. The latter enzyme often exists in greater quantities in unmalted than in malted grain, and might perhaps be thought of as essentially the amylase of mature, resting tissue.

Thorne, Emerson, Olson, and Peterson (1945) have presented evidence that amylase determinations are helpful, but not entirely adequate for the evaluation for alcohol production. The latter could probably best be obtained by fermentation tests since the limiting factor in alcohol yield appeared to be the action of the malt rather than the fermenting power of the yeast.

The present communication presents a study of the concomitant saccharifying action of malts and supplementary sources of beta-amylase. The experimental limits within which the saccharifying activity can be expressed linearly and a comparison of calculated and found values for this activity have been investigated. The possible relationship between nonamylolytic enzymes present and discrepancy from calculated values has also been investigated. Finally the effectiveness of these supplements as sugar producers (when used in equi-amylolytic amounts) has been compared with their effectiveness in alcoholic fermentations.

Methods and Materials

Alpha- and beta-amylase were determined by the methods of Olson, Evans, and Dickson (1944). The definitions used by these workers are based upon "specific" activity rather than "total" activity. Thus the "maltose equivalent" is defined in terms of a definite dry weight of malt. In actual practice this value is calculated as (blank-titration) $\times$ 144, the titration being that of a 5 ml digestion mixture aliquot (taken from a total of 200 ml) in 10 ml of 0.05 *N* ferricyanide when titrated with 0.05 *N* thiosulfate. Under their conditions this aliquot contains 1.25 mg of malt. For the purposes of the present work it is desirable to transpose this "specific" activity into a unit which does not infer weight of enzyme preparation or source used. This can be accomplished by defining one unit of enzyme as that amount of enzyme which will cause a change in titration of 1/180 ml.

$$\text{Amylase units} = (\text{blank-titration } (\Delta)) \times 180$$

When defined this way, amylase units per mg of (dry) malt become

identical with the maltose equivalent:

$$\text{Maltose equivalent} = \text{amylase units per mg} = \frac{\Delta \times 180}{1.25} = \Delta \times 144$$

Maltase and glucose were determined by the method of Schwimmer (1945), phosphatase by the hydrolysis of nitrophenol phosphate (Axelrod, 1947), and phosphorylase by the method of Green and Stumpf (1942).

Fermentation was carried out according to the procedure of Thorne *et al.* (1945) except that the autoclaved mash was cooled by the addition

TABLE I

AMYLASES, NITROGEN, AND MOISTURE OF MALTS FROM DIFFERENT BARLEY VARIETIES AND OF POSSIBLE SUPPLEMENTARY DIASTATIC SOURCES

Variety or selection	Location grown	Moisture	Nitrogen (dry)	Diastatic power		Maltose equivalent		Ratio Beta / Alpha
				°L	Maltose equivalent	Beta-amylase	Alpha-amylase	
		%	%					
M2329 Kindred	Minnesota	8.18	2.33	254	1015	895	120	7.5
A.M. 11 Norwegian	North Dakota	7.93	2.27	208	832	687	145	4.7
A.M. 2 Ezond	Nebraska	8.38	2.79	177	706	650	56	11.6
A.M. 1 Atlas	California	8.40	1.72	68	274	224	50	4.5
Commercial I	Wisconsin	8.54	1.80	93	376	348	28	12.4
Commercial II	Wisconsin	8.48	2.43	175	698	664	34	19.5
Commercial III	California	8.10	1.64	63	252	214	38	5.6
Commercial IV	California	8.39	1.68	81	322	236	86	2.7
Flour[1]	California	12.30	2.85	158	632	632	0.04	—
Flour[2]	California	—	—	43	172	—	—	—
Sweet potato (whole)	California	72.9	0.73	188	750	750	0.03	—
Sweet potato juice (dry)	California	10.43	2.75	1300	5200	5200	5	—

[1] Sulfite extract of flour.
[2] Water extract of flour.

of ice water, while the mash was subjected to the action of a "blendor." Yeast was obtained from the fermentation vats of a nearby distillery.[2]

Four of the eight barley malts used in this investigation were well-characterized barley strains [2] and four were commercial malt samples, two of which were California malts and the other two Wisconsin malts (Table I).

Samples of high protein patent flour and market sweet potatoes were used as supplementary diastase sources. For the study of saccharifying activities, a 10% flour suspension in 0.05% $NaHSO_3$ was incubated at 30° for 1 hour, and then centrifuged, the residue being

[2] We wish to thank D. F. Logan of the Hedgeside Distillery, Napa, California, for generous supplies of yeasts and malts, and Dr. A. D. Dickson of the Malt and Barley Laboratory, University of Wisconsin, for the samples of well-characterized malt varieties.

discarded. A preparation from sweet potato was made by squeezing mashed sweet potatoes through cheese cloth, centrifuging down the insoluble residue, and drying the resultant juice *in vacuo* while frozen.

In the fermentation tests, hard red winter wheat was used as source of starchy material. As a diastatic supplement, either a suspension of flour in sulfite solution was added to the malt slurry (when flour was used) or homogenized whole sweet potato was used in the fermentation tests (Table I).

Departure of Starch Conversion from Linearity with Increasing Enzyme Concentration

Comparative experiments on the concomitant action of supplement can be made if one either allows the conversion to proceed in those regions where change in enzyme concentration bears a linear relation-

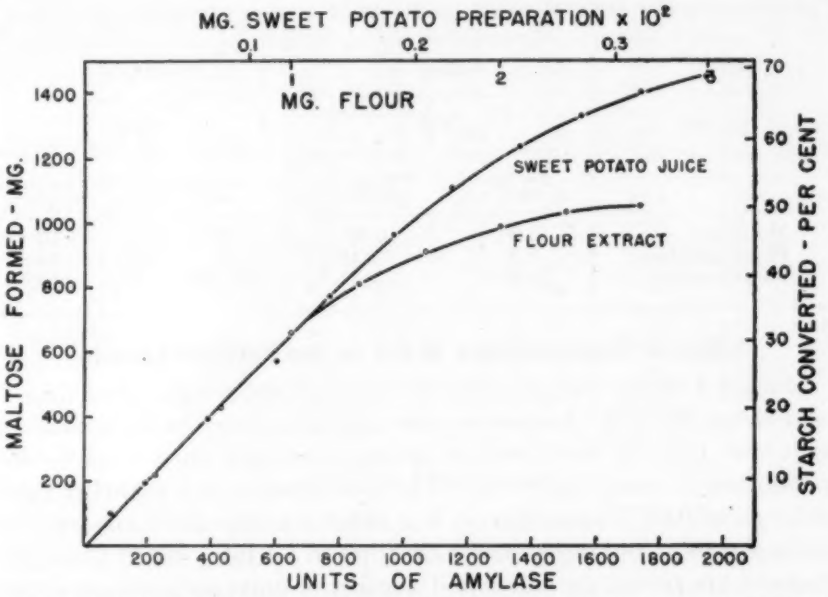

Fig. 1. Relationship between enzyme concentration and extent of starch conversion by sulfite extract of flour (open circles) and dried water-soluble extract of sweet potato juice (closed circles). The enzyme sources are given as mg per 5 ml total digestion mixture. The maltose is that formed from 2 g starch.

ship to sugar formed or if one can determine beforehand the deviation from linearity. The latter procedure is unsatisfactory, for, as can be seen in Figure 1, the deviation from linearity occurs at different stages of digestion for each source of amylase. Thus Kneen and Sandstedt (1941) found that the amylase of wheat shows linear digestion to about 30%, whereas with malt this linearity extends to 40%. This difference, confirmed for wheat in Figure 1, is due to the presence of alpha-amylase

in the malt. Since sweet potatoes contain practically no alpha-dextrinogenic activity (Table I), it is rather surprising to find a linear relationship up to the extent of about 50%, a circumstance which would indicate the participation of nonamylolytic enzymes, possibly of the oligosaccharase or the phosphate transferring classes. Some glucose was found at later stages of conversion for sweet potatoes but not for flour nor for barley malt. Table II consists of a summary of data showing the relative concentrations of maltase, acid phosphatase, and phosphorylase present in wheat and sweet potatoes. It can be seen that wheat contains no maltase but appreciable though small quantities of phosphatase and phosphorylase. On the other hand, the dried sweet potato juice is comparable in maltase strength to takadiastase (Schwimmer, 1945) and is quite high in phosphatase activity.

TABLE II

CONCENTRATION OF SOME NONAMYLASE ENZYMES IN SWEET POTATO AND IN FLOUR

Enzyme	Flour		Sweet potato	
	Units		Units	
	Per mg (dry)	Per unit of amylase	Per mg (dry)	Per unit of amylase
Maltase	0.00	0.00	0.04	8×10^{-6}
Phosphatase	0.3	4.9×10^{-4}	25	48×10^{-4}
Phosphorylase	2×10^{-4}	3×10^{-7}	26×10^{-4}	5×10^{-7}

Effect of Supplementing Malts on the Saccharogenesis

Figure 1 shows that in order to interpret the results when flour is used as supplement, the enzyme concentration must be kept within a limit such that the total amylase present must not cause a conversion amounting to more than 33%. This corresponds to a titration value of 3.6 ml of 0.05 *N* thiosulfate. For sweet potatoes the corresponding limits are 50% conversion and a titration of 5.4 ml. When these precautions are taken, the calculated number of units corresponds within experimental error to the observed results when flour is used as supplement (Table III). The correspondence between units calculated and units found for sweet potatoes is not valid for all the malts tested (Table IV). If any general inference is to be drawn concerning the lack of correspondence between found and calculated values, one may conclude that the found values tend to be higher for malts having low beta to alpha ratios (Table I). These findings are in accord with the supposition that there is present in sweet potato a system of glycosidases capable of degrading the lower dextrins and sugars formed by alpha-amylase action.

TABLE III

ADDITION OF SULFITE EXTRACT OF FLOUR AS SUPPLEMENTARY DIASTATIC SOURCE—APPARENT AMYLASE VALUES OBTAINED BY CONCOMITANT ACTION OF MALT AND SUPPLEMENT

Malt	Digestion mixture		Units (calculated)			Maltose equivalent apparent (calculated)	°L apparent	
	Malt	Supplement	Malt	Supplement	Total		Calculated	Found
	mg/5 ml	*mg/5 ml*						
Norwegian	0.625	0.173	520	109	629	1005	251	243
Ezond	0.625	0.173	441	109	550	881	220	222
	0.625	0.259	441	163	604	966	242	240
Atlas	1.250	0.345	342	217	559	448	112	109
	0.625	0.345	171	217	388	621	155	147
	0.625	0.528	171	326	497	795	199	198
	0.625	0.690	171	433	604	966	242	244
Commercial I	1.250	0.345	470	217	687	550	137	136
	0.625	0.345	235	217	452	720	180	172
	0.625	0.528	235	334	569	911	228	219
	0.625	0.690	235	436	671	1072	268	275
Commercial II	0.625	0.173	436	109	545	873	218	220
	0.625	0.259	436	164	600	960	240	241
Commercial III	1.250	0.345	315	217	532	426	106	108
	0.625	0.345	158	217	375	600	150	152
	0.625	0.528	158	334	492	788	197	188
	0.625	0.690	158	436	594	951	238	240
	0.625	0.862	158	544	702	1122	281	273
Commercial IV	1.250	0.345	402	217	619	496	124	122
	0.625	0.345	201	217	418	669	167	168
	0.625	0.528	201	334	535	856	214	208
	0.625	0.690	201	436	637	1018	254	247

Alcoholic Fermentation

To obtain comparable results on the increased efficacy of fermentation due to the presence of supplement, the grain bill was calculated so that the weight of dry matter and the total amylase were identical in each case (Table V), the amylase being brought up to the level of that present in a 5% malt mixture of the most powerful malt used, *Kindred*. When the fermentation is allowed to proceed for 24 hours in the manner described, it can be seen (Table VI) that sweet potato (on an equi-amylolytic basis) is a more efficient supplement for alcohol production than is flour. Furthermore the three highest malts, whether beta-supplemented or not, gave the best alcohol yields. Again this is in accordance with the previous observations on the synergistic nature of the concomitant action of high alpha-amylase malt and sweet potato. As expected, there is no obvious relation between the Lintner values of

the malts and alcohol yields in the presence of supplements. In no case did the yield exceed that obtained with the "reference" malt, *Kindred*. It is of interest to note that the malt labeled "Commercial IV," possessing a very low beta to alpha ratio, performed most efficiently of all the malts used in the presence of supplement.

TABLE IV

ADDITION OF DRIED SWEET POTATO JUICE AS SUPPLEMENTARY DIASTATIC SOURCE—APPARENT AMYLASE VALUES OBTAINED BY CONCOMITANT ACTION OF MALT AND SUPPLEMENT

Malt	Digestion mixture		Units (calculated)			Maltose equivalent apparent (calculated)	°L apparent	
	Malt	Supplement	Malt	Supplement	Total		Calculated	Found
	mg/5 ml	*mg/5 ml*						
Norwegian	0.625	0.02	520	104	624	998	249	265
Ezond	0.625	0.02	441	104	545	872	218	217
	0.625	0.03	441	156	597	955	239	245
Atlas	1.250	0.04	342	208	550	440	110	109
	0.625	0.04	171	208	379	606	151	128
	0.625	0.06	171	312	483	772	193	202
	0.625	0.08	171	416	587	940	235	222
Commercial I	1.250	0.04	470	208	678	542	136	138
	0.625	0.04	235	208	443	709	177	177
	0.625	0.06	235	312	547	875	219	224
	0.625	0.08	235	416	651	1042	261	255
Commercial II	0.625	0.03	436	156	592	947	237	228
	0.625	0.05	436	260	696	1130	282	258
Commercial III	1.250	0.04	315	208	523	418	105	103
	0.625	0.04	158	208	366	585	146	145
	0.625	0.06	158	312	470	752	188	189
	0.625	0.08	158	416	574	918	230	236
Commercial IV	1.250	0.04	402	208	610	488	122	135
	0.625	0.04	201	208	409	654	164	172
	0.625	0.06	201	312	513	821	205	210
	0.625	0.08	201	416	617	988	247	271

TABLE V

USE OF SUPPLEMENT FOR FERMENTATION—SUPPLEMENTARY QUANTITIES REQUIRED FOR EQUIAMYLOLYTIC MASHES

Malt variety	Percent of grain bill due to		
	Malt	Malt + flour	Malt + sweet potato
Kindred	5.00	(5.00)	(5.00)
Norwegian	5.00	6.45	6.22
Ezond	5.00	7.45	7.07
Atlas	5.00	10.86	9.96
Commercial I	5.00	10.06	9.26
Commercial II	5.00	7.52	7.11
Commercial III	5.00	11.04	10.10
Commercial IV	5.00	10.48	9.62

TABLE VI

USE OF SUPPLEMENTS FOR FERMENTATION—ALCOHOL YIELDS FOR 24 HOURS, PROOF GALLONS PER 100 POUNDS

Malt variety	Malt	Malt + flour	Malt + sweet potato
Kindred	9.5	(9.5)	(9.5)
Norwegian	8.1	8.8	9.3
Ezond	7.6	6.9	8.5
Atlas	6.4	7.1	8.1
Commercial I	6.8	—	8.4
Commercial II	7.4	8.3	8.5
Commercial III	6.4	—	8.9
Commercial IV	7.7	9.2	9.5

Summary

A study has been made of the concomitant saccharifying action of various barley malts in the presence of sweet potatoes or flour, each as a supplementary source of beta-amylase. The limits of enzyme concentrations within which the rate of reaction is proportional to the enzyme concentration and within which valid comparison of this action can be made have been determined. For flour, this limit is that amount of enzyme which will cause not more than 30% starch conversion under the stated conditions and not more than 50% for sweet potato amylase. Within these limits, it has been found that the calculated activity of malt and flour mixtures is about the same as the experimentally determined activity, whereas the latter value tends to be higher than that calculated for mixtures of sweet potato and malts high in alpha-amylase activity. When mixtures of malts and supplement are used in fermentation tests in equiamylolytic amount, the subsequent yield of alcohol is greater for the sweet potato supplemented mashes. These results, which consistently demonstrate more extensive action in the presence of sweet potato, are consistent with the demonstration of appreciable concentrations of nonamylolytic enzymes therein concerned with sugar transformation.

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ANNOUNCEMENT

Bibliography and Review of the Scientific Literature on the Deterioration of Fats and Oils. By B. F. Daubert, Quartermaster Food and Container Institute, Chicago, Illinois.

The Committee on Food Research, Office of the Quartermaster General, is making available a bibliography and review of the scientific and technological literature related to the deterioration of fats and oils, prepared by Dr. B. F. Daubert, Research Professor, University of Pittsburgh.

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Tricalcium Phosphate
Calcium Pyrophosphate
Sodium Iron Pyrophosphate
Iron Orthophosphate
Iron Pyrophosphate

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Experimental samples on request

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